MICRO-ETCHED PLATFORMS FOR THERMAL INACTIVATION OF *BACILLUS ANTHRACIS* AND *BACILLUS THURINGIENSIS* SPORES

THESIS

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AFIT/GWM/ENP/08-M01

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Abstract

This research developed and applied microlithography techniques to etch microscope slide platforms in order to measure thermal inactivation of *Bacillus thuringiensis* and *Bacillus anthracis* spores, two closely related *Bacillus* species. *B.t.* is widely used as a surrogate for *B.a.* in response studies and in some field studies. This work addressed the previously identified problem of measuring both spore growth and spore thermal kill threshold using the traditional method of spreading a diluted spore solution on a plate. The micro-etched platforms forced spore separation thereby preventing neighbor growth from obscuring germination and initial vegetative growth measurements using a microscope. The technique permits observation of small samples of spores over time and yielded more accurate response measurements. This study includes comparison of thermal responses between *B.a.* and *B.t.* spores that were prepared and stored in exactly the same environment and conditions. Findings support the continued use of *B.t.* as a substitute for *B.a.* in this type of work and especially in studies for thermal inactivation for short times periods of a minute or less. The micro-etched slides can also be applied to laser inactivation of spores for exposure times as short as milliseconds.

Acknowledgements

During the course of this research I had the pleasure of working with a great number of professionals in many disciplines at the Air Force Institute of Technology. Some were professors, others were fellow student, but most were supporting technicians. Despite their variety of expertise, the common threads among them was that they were all readily available, willing to help, generous with their time, and supportive of my efforts.

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Of course foremost in the development of this research, I wish to thank my advisors, Dr. Larry Burggraf and Dr. Charles Bleckmann. Both gentlemen are approachable and extremely well versed in their fields. Dr. Burggraf's passion for learning coupled with the patience necessary to allow students to explore their own path to knowledge is unique to this level of academics. Dr. Bleckmann's willingness to be my sounding board as I dissected problems and his quiet strength imparted the confidence that I needed to work through obstacles. The remaining members of my committee, Dr. William Baker and Dr. Guangming Li, readily shared their knowledge of lasers, material properties, and mathematical models during the course of my work.

Finally and most important of all, I wish to thank my family for all their emotional support and the sacrifice of time that allowed me to accomplish this goal.

Leslie S. Hawkins

TABLE OF CONTENTS

Page

Page

List of Figures

Figure Page

List of Tables

Table Page

MICRO-ETCHED PLATFORMS FOR THERMAL INACTIVATION OF *BACILLUS ANTHRACIS* AND *BACILLUS THURINGIENSIS* SPORES

I. Introduction

Background

The realities of current world political and ideological thought ensure the continued trend of asymmetrical warfare tactics. Despite more than a century of global attempts to eradicate the use of Chemical and Biological Weapons (CB), the threat of their wide spread employment on civilian targets has increased. Indeed, the well known biological agent Anthrax, *Bacillus anthracis* (*B.a.*), was recently used to terrorize American citizens in the 2001 discovery of *B.a.* spores sent through the US postal system to two senators. Homeland security concepts are clearly focused to defend the average citizen against such potential CB attacks by both foreign and domestic terrorist organizations. This research supports the interest of both civil and military organizations in pursuit of neutralization of anthrax. *Bacillus anthracis,* because of its spore formation, may be the most difficult weaponized biological agent to destroy by conventional or incendiary device.

Combating the threat of the use of CB agents is the focus of many agencies in both the government and civilian sectors. Algorithms are being developed to try to predict the reaction and neutralization of several of the known agents using variations of both conventional and unconventional weapons scenarios. These algorithms must address agent response to a variety of environmental stressors to include temperature, humidity, radiation, and chemical reactions. However, additional experimental data is needed as input to these algorithms to assure accurate predictions.

The subject of this project is *Bacillus* spore, specifically the *Bacillus anthracis* Sterne strain (*B.a.*) and *Bacillus thuringiensis* var. *kurstaki* (*B.t.*). It is common in experiments to use nonpathogenic members of *Bacillus* as simulants, since they are genetically similar. *B.t.* and *B.a.* differ in the presence of peptides in *B.t.* that presumably prompt the formation of crystalline coating over the spore coat. Although *B.t.* is used as a *B.a.* simulant, the *B.a.* Stern strain is not because it can induce a biochemical reaction in humans. Since *B.t* species is used as a simulant, a side by side comparison of the two species and their response to heat in necessary to confirm whether this substitution is accurate for thermal inactivation studies.

Currently, there is abundant research data available concerning the response of *Bacillus* spores to high temperatures over minute and hour time frames, but little data on spore reaction to high heat in a time event of a second or less (Beaman, Greenamyer, Corner, Pankratz, & Gerhardt, 1982; Spotts Whitney et al., 2003; Turnbull, Frawley, & Bull, 2007). The focus of this research is on the application of heat to the bacterial spore during very small time intervals of a minute or less which corresponds to counter proliferation scenarios. The intent is to gather response data at these shorter times to lay the foundation for further research to develop response parameters for even shorter exposure times of less than a second. The ultimate goal is to confidently predict a spore's response to a real world exposure to the fireball created by the fire produced by an incendiary weapon or the detonation of a conventional weapon.

The time-temperature spectral phenomenon profiles of a conventional high explosive fireball are currently being investigated by a research group lead by Dr. Perram at AFIT. Dr. Kevin Gross, also at AFIT, recently developed a seven-parameter model

(fireball size, temperature, particle absorption coefficient, gas concentrations for H_2O, CO_2, CO, and HCl, of mid-wave infrared fireballs that correlate with the physical events by both type and weight of the high explosive. (Gross, Kevin C. 2007). The peak fireball temperatures can be fit to a Plankian function with the assistance of a Matlab code written by Dr. Gross. For an agent defeat weapon, RDX explosive, the peak temperature of 1578 ºKelvin was reached at 0.5 seconds with the heat from the fireball lasting a total of 4 seconds (Orson, J.A. 2003; 104). From this data, the short time-temperature kill threshold for conventional weapons that we eventually want to determine is up to 1600 ºKelvin for a period of less than 4 seconds.

Simply stated, the question at hand is whether a stock pile of weaponized anthrax spores can be confidently destroyed by a conventional weapon. This research will be used to explore and identify the response of *Bacillus anthracis* spores to high temperatures over extremely short time durations of seconds in efforts to determine lethality thresholds. Because of the historical and practical substitution among the *Bacillus* species in experimentation, this research also includes work to validate this practice at shorter exposure time durations.

This project is a direct continuation of earlier effort of another AFIT student, Kristine Goetz. The focus of her work was to develop a short time heating method to measure spore kill probability. Goetz developed and tested the use of a Nd:YAG laser to indirectly heat spores dried on a microscope slide. Silicon-carbide sandpaper was mechanically clamped to the underside of the spore-laden microscope slide. The laser was then moved across the spores at various velocities using several laser power levels. The silicon-carbide sandpaper backing absorbed infrared radiation producing a miniature

fireball. The heat localized in the area beneath the laser track thereby heated the spores to a specific temperature for short time intervals. (Goetz, 2005) The temperature of the miniature fireball was measured using FTIR spectroscopy in the same manner that Dr. Gross measured temperatures profiles of conventional munitions. (Gross, 2007)

Purpose and Approach

The additional contribution of this project to earlier work is a new spore slide design that seeks to resolve some of the following lingering issues for this method that were identified by Goetz. These issues include the ability to accurately determine colony counts to measure kill probability and confidence in accurate time and temperature exposure for each spore. (Goetz, 2005) The difficulty centered on the use of a standard dilution of spores that were then spread on a plate for counting. The spacing of the spores was inherently irregular on the plate. Because of this distribution, it was difficult to determine the exact temperature exposure to each spore. This issue was compounded by the fact that the laser line itself experiences its own temperature profile. Although the peak laser temperature was adequately defined along the center, the spores outside this center were exposed to fractions of the peak power which was much harder to determine. Furthermore and most important for this work was the fact that once the spores germinated, areas of congestion experienced overlapping of vegetative cells obscuring the ability to make accurate visual counts.

The small laser spot size dictates that improvements to this method still employ a microscopic count method to verify a short time temperature profile. In order to do this, spores must be separated by a distance greater then or equal to the size of the largest

expected colony growth at the time of the measurement. The spore separation must also account for the laser beam's inherent temperature profile.

The focus of this research was to gather enough data to statistically verify the lethality profile of *B.a.* and *B.t.* spores exposed to a series of time and temperature profiles using an optical microscope method consistent with the laser killing method developed by Goetz. This was done by accomplishing a series of smaller milestones: (1) design and manufacture a slide surface that separates spores; (2) develop heating procedure to expose the separated spores to high temperatures within the limited duration of under a minute; (3) compare the thermal susceptibility of *B.t.* compared to *B.a.* at these short time exposures; (4) determine spore survival rate dependence on heat exposure time; (5) and finally to verify ability to repeat experiment and gain enough data to statistically support conclusions. An additional advantage by achieving theses goals was found to be the capacity to measure spore germination times and outgrowth of the survivors.

The start point was the design and manufacture of glass slide platform that supported both colony counting needs and limited exposure uncertainty. The traditional spore viability test methods involve the dilution of spores on a standard count plate. There are several acknowledged limitations with this method. First, estimations are made in the amount of spores present since they are numerous in the solution. Secondly, counting colonies once germination begins is a very laborious undertaking. Not only are the spores small, approximately 1 micron in diameter, but once germination occurs the resulting overgrowth and matting quickly obscures the field of view for the observer.

The planned approach to address these issues in this work was to place the spores on a slide specifically designed to separate spores. Divots or holes were chemically etched into glass slides using a microlithography technique. The size of the divots was about 3 by 3 microns with a depth of about a micron. They were placed on a regular grid pattern. The concept was a simple one. The spores were spread out over the surface of the developed grid where the spores found a place in each divot like an egg-carton. Excess spores were swept from the higher surface.

The separation distance of spores in their respective rows addressed the problems of colony counting. This technique alleviated the practice of estimating spores present on the slide. In essence, one hole was filled with a limited number of spores. Ideally that number would be limited to only one spore per hole, but that goal was never repeatedly achieved in this experiment. The set distance also prevented the visual interference caused by overgrowth by giving each spore ample room to germinate. The best method to encourage the spores to both find and settle into a divot and removal of the excess surface spores was identified after numerous experiments.

Heating of the spore laden platforms was accomplished using a small ceramic furnace. A silicon nitride base provided a uniformly heated surface which ensured a uniform temperature exposure for the entire platform surface. Surface temperature was measured using a thermocouple. The minimum time exposure was 5 seconds due to the physical limitation presented by this manually operated system. The times of thermal diffusion through the 0.17 millimeter thick platform was very fast compared to the exposure time.

This method of heating was used throughout this experiment. First, both *Bacillus* spores were exposed to different temperatures and times to determine their kill threshold for that particular spore preparation. Once separate data was collected for each species, the results were compared to verify their heat response comparability. After that, the micro-etched platforms were used to more closely measure the heat induced response of *Bacillus anthracis* spores as they approached the predetermined kill threshold point.

II. Literature Review

The Genus *Bacillus*

The members of the *Bacillus* genus can be readily found in nature, most notably, in a wide variety of soils to include hot or cold, acidic or alkaline, desert or fertile or even in the floors of both salt and fresh water bodies. *Bacillus* are gram positive, rod- shaped aerobic organisms. (Logan & Turnbull, 2003: 445) When they grow they form colonies between 2 and 7 millimeters in diameter (Logan & Turnbull, 2003: 454). The common characteristic of all members of the *Bacillus* genus is that, in the presence of oxygen, they will produce endospores. These endospores become the inactive, dehydrated cells which are resistant to environmental conditions.

Bacillus anthracis

Bacillus anthracis is well known for several reasons. First, it was isolated in 1877 by Robert Koch and used to establish his postulates for defining a causative relationship between microbes and disease. (Willet, Joklik, Amos, & Wilfert, 1992: 633) Later in 1881, Louis Pasture tested his live bacterial vaccination principles using *Bacillus anthracis*. (Mock & Fouet, 2001:661) Lastly and most notoriously, *Bacillus anthracis* is the etiological cause of the disease known as anthrax. Anthrax is generally a disease known to infect grazing animals. However, humans can contract it through close contact with these herbivores' hide, wool or fur. Anthrax has been suspected as the source of several plagues throughout history and may be the Sixth Plague mentioned in the Biblical Book of Exodus (Chapter 9, Verses 8-12). More recently, anthrax has been weaponized

for use as a biological weapon. The first recorded use as a weapon was in 1915 when German agents in the U.S. injected horses, mules and cattle being shipped to Europe with the anthrax bacterium. There is evidence that Japan tested anthrax as a biological weapon against humans is 1937. The United Stated started development of anthrax as a biological weapon in 1943. (Anonymous; Weaponization of Anthrax)

When the bacteria begin to grow, they form a series of rods that appear squared at the ends. These rods are about 1 to 3 microns in diameter and between 5 and 10 microns in length. (Willet et al., 1992:633) Ellipsoidal spores are formed when the vegetative cells are exposed to special conditions causing sporulation. Figure 1 below shows a gram stained sample of *B.a*. Note the forming endospores within some of the vegetative cells.

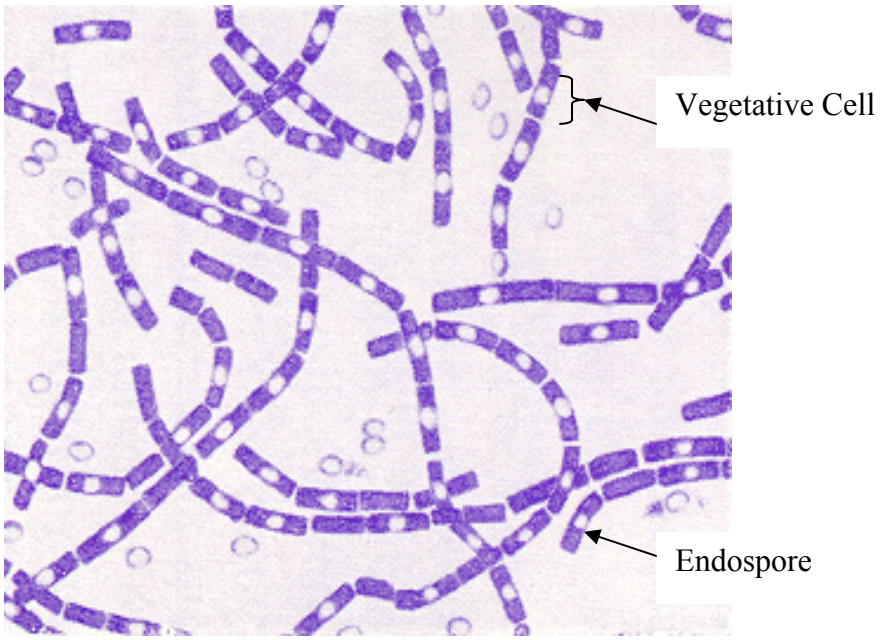

Figure 1. Gram strain of *Bacillus anthracis* 1500X. The cells have characteristic squared ends. The endospores are ellipsoidal shaped and located centrally in the sporangium. The spores are highly refractile to light and resistant to staining. // textbook of bacteriology.net

Bacillus thuringiensis

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Bacillus thuringiensis is also a well known member of the *Bacillus* genus. After its vegetative cell reaches maturity, it forms a rhomboidal crystalline protein as a natural by-product. It was discovered in the early 1950s that this protein is potent toxin to silkworms and caterpillars (Gould & Hurst, 1969: 495-509). This toxic effect to insects made it a candidate for use in natural insecticides. It continues to be a popular additive to many pest control formulas. Compared to its cousin *B.a*., it is slightly smaller. The vegetative cells of *B.t*. are approximately 1 micron wide and 5 microns long.

The Bacterial Spore

In response to an unfavorably environment, both *Bacillus anthracis* and *Bacillus thuringiensis* cells achieve a dormant structure commonly known as a spore. While in this state the bacteria display a distinct lack of metabolic activity. In this state the organisms are extremely resistant to changing environmental factors. Studies have shown resistance to such factors as temperature, pressure, ultraviolet (UV) radiation, and even some chemical agents. Although little activity takes place, there is evidence of some responses to environmental changes that cause the spore, while still dormant, to change shape, size, flexibility, or chemical composition. Also while in this dormant state the spore remains ready to grow again when conditions become favorable. Research suggests that the spores possess "an alert sensory mechanism which is able to respond to specific germinants within minutes" when circumstances present themselves allowing for germination (Leuschner & Lillford, 2001: 36) which implies that some of the proteins are functional even in this dormant state.

The Spore Structure

A series of concentric shells compose the protective coating surrounding the spore. These layers protect the genetic material in two ways. First they lock the DNA in an inactive, crystalline, desiccated, and stable state. This desiccation contributes to heat resistance and dormancy of the spore. Second, the hard outside shells serves to prevent toxic molecules from reaching the encased DNA. (Driks, 2003:3007). Figure 2, below, is an illustration of this structure.

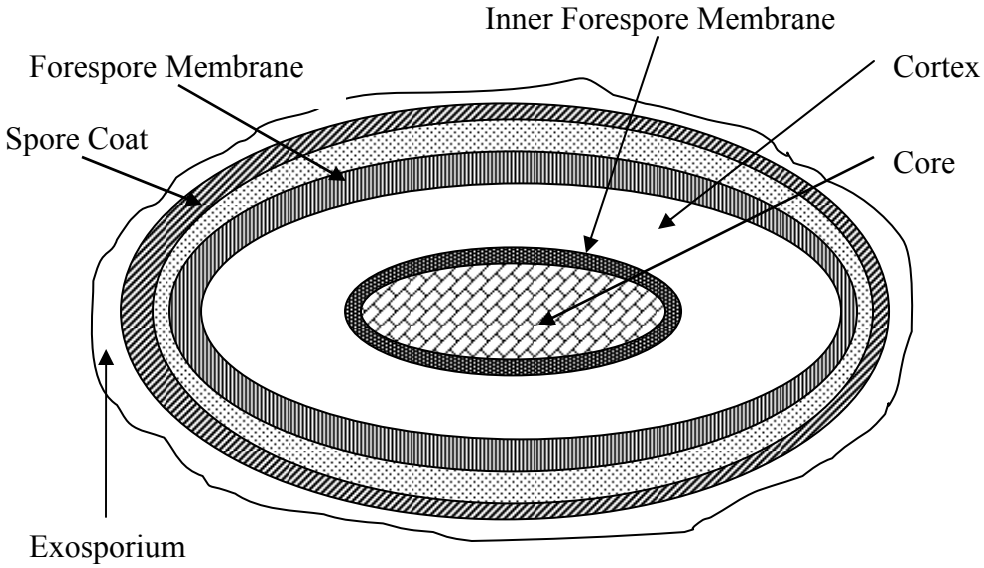

Figure 2. Structure of a *Bacillus* Spore. The spore can be broken down into a series of protective layers. Each layer plays a part in protecting the dormant spore by restricting the movement of water molecules and keeping toxic molecules from reaching the DNA housed in the core.

The outermost layer of the spore is called the exosporium. The major components of the exposporium are protein, lipid, and carbohydrate (Mock & Fouet, 2001:651). However, very little is known about its function. Since some members of the *Bacillus*

genus do not have an exosporium (e.g. *Bacillus subtilis*) but are nevertheless resistant to the same environmental stressors it is hypothesized that its role is more niche accommodation than protection. (Driks, 2003:3007). Indeed both *B.a*. and *B.t.* germinate without an exosporium. In support of this accommodation hypothesis, filamentous appendages called pili can cover the surface which are thought to aid in spore attachment (Mock & Fouet, 2001:651).

The spore coat lies directly beneath the exosporium (Driks, 2003: 3007). This region serves as a barrier against large toxic molecules. It is composed of two distinct layers. The outer layer is thick while the inner layer is banded. The coat has been shown to include over 25 proteins (Driks, 2003: 3008). These proteins may also play a role in germination (Babyan & Setlow, 2002: 1219).

Delving deeper is a layer known as the forespore membrane (Driks & Setlow, 2000: 193). Like the exosporium, little is known about this membrane and its function.

Next is the cortex. The cortex is a tight fabric that keeps the DNA dry by forming a boundary layer around the core. This boundary allows small molecules, like water, to pass through. The core remains essentially dry, however, because of the constricting action of the cortex. What little water that remains in the cortex plays a part in the spore's susceptibility to thermal damage. The peptidoglycan strands found in this region allow the cortex to both expand and contract in response to ionic and pH changes sensed by the spore (Driks, 2003: 3007). It must be noted that a thin layer of this peptidoglycan will become the initial vegetative cell wall once germination occurs (Popham, Helin, Costello, & Setlow, 1996: 15405).

Lastly, the final barrier is referred to as the inner forespore membrane. Almost

nothing is known about this final boundary before the core. The core is where the spore maintains its genetic material to include the nucleoid, enzymes, and ribosomes (Driks & Setlow, 2000: 193). Several small acid-soluble proteins also reside within the core and protect the DNA from heat and UV radiation (Driks, 2003 3007).

Sporulation

The depletion of nutrients such as carbon or nitrogen, prompts *Bacillus* to form spores (Setlow & Johnson, 2001:34). These spores grow readily in a culture, natural soil, and animal carcasses. (Willet et al., 1992) Sporulation can be divided into eight stages. All of these stages can take place within eight hours. A brief discussion of these stages follows. For clarification it must be noted that the term "endospore" is used at the initial point when the spore is being constructed inside the vegetative cell (Setlow & Johnson, 2001: 35).

The eight stages begin with the vegetative cell at Stage 0. Here chromosomes are replicated into two separate nucleoids. (Setlow & Johnson, 2001: 36). An axial filament is then formed from these two nucleiods in Stage I.

Next, in Stage II, the development of the forespore is initiated with the formation of a double membrane septum which divides the cell in two with each half receiving one of the two nucleoids. The two cells consist of a mother cell and the new forespore, with the forespore being the smaller of the two portions. A further division will eventually occur and the outer of the two membranes become the forespore membrane, while the inner will become the inner forespore membrane once the spore is fully formed (Setlow & Johnson, 2001: 36). The nucleoid inside the new forespore condenses on its way to

becoming the spore core.

At Stage III, the mother cell engulfs the forespore and the prespore protoplast is formed. Next, in Stage IV, the cortex is formed. Peptidoglycan is deposited between the double membranes. It is during this stage the dehydration within the spore begins (Setlow & Johnson, 2001: 36). The space between the membranes will become the fully formed cortex. The germ cell wall forms between the cortex and the inner forespore membrane.

At Stage V (coat formation), an inner spore coat protein is deposited on the surface of the outer forespore membrane (Setlow & Johnson, 2001: 36). Next in Stage VI the spore matures and deposits another outer spore coat on the surface of the inner spore coat (Setlow & Johnson, 2001: 37). The spore now has accumulated the dipicolinic acid in the spore core which is synthesized by the mother cell (pyridine-2,6-dicarboxylic acid or DPA) (Setlow & Johnson, 2001: 37). The dipicolinic acid protects the spore DNA from extreme environmental stresses.

At its core the spore is dehydrated. The permeability of the membranes is set. Dormancy is reached. The final stage is when the mother cell lyses and releases the mature spore into the environment (Setlow & Johnson, 2001: 37). Below is a depiction of this eight stage process, Figure 3 (Setlow & Johnson, 2001: 35; and Driks & Setlow, 2000: 191-210).

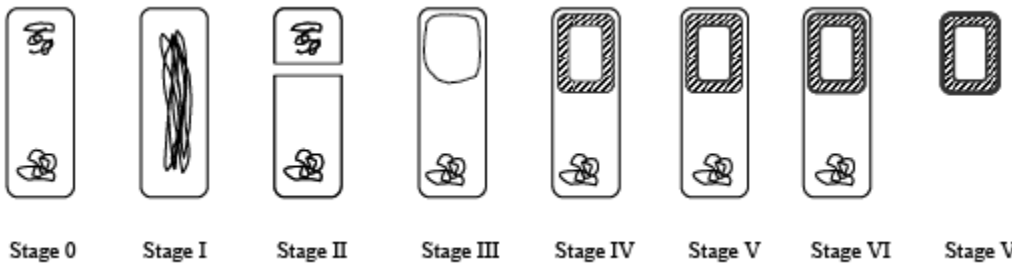

Figure 3. Stages of Sporulation (Goetz, 2005:13). Depletion of nitrogen or carbon results in the vegetative *Bacillus* cells forming an endospore inside the cell itself. The cell forms this endospore in a series of eight distinct stages that replicates genetic material, divides that material, and creates the protective spore coat while dehydrating the endospore's core before releasing it into the environment.

Germination.

When the spore becomes vegetatively active again germination is said to occur. Germination has been defined as "*the change from a heat-resistant spore to a heat-labile entity which may not necessarily be a true vegetative cell*" (Halmann, M. 1962; 1187). This change is considered to be a biological response to the presence of specific chemicals such as alanine. It has been suggested that L-alanine dehydronenase might be the chemical bonding site triggering alanine deamination that might be the first step in a germination process. However, it is not known whether it is this chemical event or subsequent enzymatic reactions or a combination that actually induces germination. Observation of a physical manifestation of this response occurs after a lag period in the studied spores suggesting that germination is a result of a multi-step metabolic chain. (Halmann, M. 1962; 1187)

Like sporulation, germination can also be broken down into sequential stages. Here there are only three: activation, germination, and outgrowth. A depiction of these stages is shown in Figure 4.

Activation is triggered by heat or the presence of certain chemicals and is the only stage that is reversible if the environment reverts to a hostile state. This stage is usually prompted by mild heating of approximately 65 º Celsius for less than an hour. This activation is routinely done in laboratory spore experiments. The actual time and temperatures vary depending on each laboratory's standing procedures. Although structural changes are not directly observed in this stage, the appearance of the spore coat becomes mottled and the contained cytoplasm becomes less granular. (Moberly, Betty J. 1966; 221)

Germination terminates the dormant spore's cryptobiotic state. It occurs when the spore comes into contact with many types of nutrients as well as several non-nutrient stimulants. The result is the initiation of a breakdown of macromolecules and excretion of spore substances such as dipicolinic acid, calcium, and peptides. This stage does have observable physical events. The spore coat remains intact, but the cortex dissolves as the core swells, filling the exosporium. This is a key step in spore rehydration. The inner forespore and forespore membranes become more distinct and begin an infolding typical of an active cytoplasmic membrane. Initially, the cytoplasm and nucleoplasm are mixed, but later separate into groups of ribosomal granules and DNA strands. (Moberly, 1966; 221)

Finally, outgrowth occurs when the spore synthesizes the proteins and structural components necessary to become a vegetative cell. New macromolecules are synthesized as the new cell emerges and divides. The core wall thickens to become the vegetative cell wall. Mesonomes appear near the in growing septum that marks the dividing point. The most notable change is the elongation and enlargement of the cell. Often remains of the

outer part of the old spore coat and the exosporium are found within this new cell. Cast off vestiges of the spore can also be detected in the surrounding environment. Occasionally, fission imbalances occur and there are cells that do trisect. (Moberly, 1966; 221,226) In its vegetative cell form, the bacteria's increased heat resistance and hardiness are gone. (Willet et al., 1992: 72)

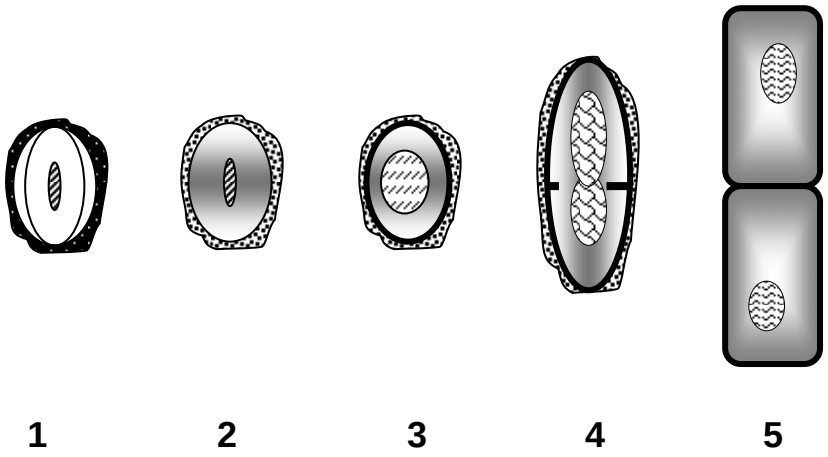

Figure 4. Illustration of Germination. (1) is the dormant spore with the internal structures as seen in Figure 2. (2) is the activation stage where the spore coat takes on a mottled appearance and the cytoplasm is more opaque. (3) is the start of the germination stage were the core swells, the cortex and inner membranes dissolve, and the cell wall thickens.(4) is the later stage of germination where the cell enlarges and elongates, the ribosomal granules and DNA strands separate and an in growing septum appears at the division point. (5) is the outgrowth stage where the septum in completed elongation is completed and vegetative cells exist.

Damaging Spores with Dry Heat

As stated earlier, bacterial spores are resistant to changes in temperature. However, damage is possible with either extremely high temperatures or prolonged low temperature exposure. By analogy with thermal inactivation of vegetative cells, threshold damage mechanism may center on hydrolysis-type DNA damage or by irreversible dehydration of the proteins that assist in germination within the spore core**.** This is

consistent with better known damage processes in fully vegetative cells. However, there is little currently known about these processes in spores.

Two types of DNA hydrolysis reactions are depurination and deamination are most important. In deamination, an amino group from one of the DNA bases is removed. For example, the removal of the amino group from a cytosine base may give rise to a uracil or thymine base instead which would pair with adenine when the original code would attract a guanine base. This change in the DNA sequence can result in future damage during DNA code replication. Thus a change in the DNA base code would both propagate and replicate as the essential cell proteins would be improperly produced or not produced at all because of this DNA damage.

Depurination is a more common event and is more harmful because it leads to a high frequency of mutations. The higher the temperature applied to the spore, the greater the probability of depurination. It occurs spontaneously, and is accelerated at high temperatures. During this event, either an adenine or guanine is severed from the deoxyribose chain leaving the chain backbone intact. Missing the base pair missing prevents the DNA strand from matching up with the other half of its double helix. The correlation of spore water content in the core and thermal sensitivity supports the postulate that the thermal inactivation threshold mechanism is DNA hydrolysis. Indeed, the water content within the spore's core decreases with higher sporulation temperatures, confirming greater thermal resistance.

The activation energy required for such DNA hydrolysis should not vary unless there was a significantly different damaging process. If there was a change in the activity of the water within the spore, the rate of hydrolysis damage changes. This model of

inactivation focuses on the change to the activity of water and is outlined below. We account for the thermal breakdown of the DNA in compounded by the stress of hydrolysis in Equation 1 below.

$$\frac{d[DNA]}{dt} = \{-k_1(T) * [DNA]\} - \{k_2(T) * [H_2O] * [DNA]\} \qquad \text{(Equation 1)}$$

where

- [DNA] is the information content of the contained DNA
- $[H_2O]$ is the water activity inside the spore coat
- $k_1(T)$ is the rate coefficient for thermal damage exclusion of hydrolysis
- $k_2(T)$ is the rate coefficient for hydrolysis damage of DNA

Since the hydrolysis reaction rate increases with increasing temperature, a potential model for thermal inactivation of these bacterial spores is based on an Arrhenius relationship. This model assumes that there is a threshold response. In other words, if thermal induced damage accumulates above a set point, the spore will die. The temperature dependence of an Arrhenius rate constant is shown below.

$$k = A * \left(e^{\left(\frac{-E_a}{RT} \right)} \right) \qquad \text{(Equation 2)}$$

where

- k is the first order rate constant
- A is the Arrhenius pre-exponential factor (which has very weak temperature dependence)
- E_a is the activation energy ($Jmol^{-1}$)
- R is the gas constant (8.314 $Jmol^{-1}$)
- T is the absolute temperature (K).

Integrating Equation 1 and representing the initial information on undamaged DNA as $[DNA]_0$ we get Equation 3 below.

$$\ln\left[\frac{[DNA]}{[DNA]_0}\right] = -\int_0^t k_1(Temp) + k_2(Temp) * [H_2O]dt \quad \text{(Equation 3)}$$

Then evaluating Equation 3 assuming a constant average water activity and temperature results in Equation 4.

$$\ln\left[\frac{[DNA]}{[DNA]_0}\right] = -k_1(Temp) + (k_2(Temp) * [H_2O]_{average} * time) \quad \text{(Equation 4)}$$

Letting $[DNA]_{kill}$ represent the a "kill' threshold point at which the accumulated DNA damage is sufficient to prevent germination, C_{kill} is defined as follows.

$$C_{kill} = -\ln\left[\frac{[DNA]_{kill}}{[DNA]_0}\right] \quad \text{(Equation 5)}$$

Only a small fraction of accumulated damage to the DNA sequence results in a high probability of kill. C_{kill} is then approximately equal to the fraction of DNA damage, because $C_{kill} = -\ln x \approx 1 - x$.

Making an additional assumption that the activation energy required for damage to DNA are approximately the same regardless of the thermal inactivation mechanism involved, then temperature and time are related to the DNA damage fraction by Equation 6.

$$\frac{1}{Temp} = \frac{R}{E_1}\ln[time] + \frac{R}{E_1}\ln\left\{\frac{1}{C_{kill}}\left(A_1 + A_2 * [H_2O]\right)\right\} \quad \text{(Equation 6)}$$

Models for Dry Heat Inactivation

A great deal of research has been done to examine the response of various *Bacillus* species to temperatures stress. However, the heat exposure times mainly exceed one minute. The data from this longer time research is valuable for trend analysis. This compilation was discussed thoroughly in the work by Goetz (Goetz, 2005).

Spotts-Whitney et al. collected a comprehensive review current literature which dealt with the inactivation of *Bacillus anthracis*. The portion of the survey which dealt with spore heating is included below in Table 1 (Spotts-Whitney et al., 2003: 624).

Table 1. Heat Inactivation of *Bacillus anthracis* Spores from Spotts-Whitney et al

Temperature	*Time*	*Inoculum Size*	*Inactivation Effect*
Boiling			
100° C	10 min	$3x10^6$	Sample Sterilized
	5 min	$7.5x10^8$	Sample Sterilized
Moist Heat			
90° C	20 min	$1.2x10^6$	Sample Sterilized
90° C to 91° C	60 min	$3x10^8$	Spores Detected
100° C	10 min	$1.2x10^6$	Sample Sterilized
100° C to101° C	17 min	$1x10^5$	Sample Sterilized
105° C	10 min	$3x10^6$	Sample Sterilized
120° C	15 min	$2.4x10^8$	Sample Sterilized
Dry Heat			
140° C	>90 min	$6x10^3$ to $1.2x10^4$	Sample Sterilized
150° C	10 min	$6x10^3$ to $1.2x10^4$	Sample Sterilized
160° C	10 min	$6x10^3$ to $1.2x10^4$	Sample Sterilized
180° C	2 min	$6x10^3$ to $1.2x10^4$	Sample Sterilized
190° C	1 min	$6x10^3$ to $1.2x10^4$	Sample Sterilized
200° C	30 sec	$6x10^3$ to $1.2x10^4$	Sample Sterilized

For dry heat the shortest time frame explored was 30 seconds. Temperatures required for sample neutralization were at least 200ºC. Goetz plotted this compilation of data from several experiments in one graph as shown in Figure 5 (Goetz, 2005:23). The

inactivation temperatures have a reasonably linear fit when heating time is on a logarithmic scale. Fernelius et al. measurement of *B.a.* survivability. While Molin and Östlund measure *Bacillus subtilis*, (*B.s.*) (Molin & Ostlund, 1976: 557). However, as discussed earlier, each species of *Bacillus* spores should exhibit very similar resistance to heating, so it is assumed that their results would be comparable to work done using *B.a.* These variations in temperature responses may be attributed to varied sporulation conditions of a particular *Bacillus* organisms. Heat resistance of spores can be greatly affected by environmental conditions during the sporulation process. Temperature, humidity, and time spent at each sporulation stage can influence a spore's ability to withstand heat (Faille et al, 2002: 1930-6). For this reason, all the conditions ranging from storage to incubation of the *B.a.* and *B.t.* spores in this research were identical.

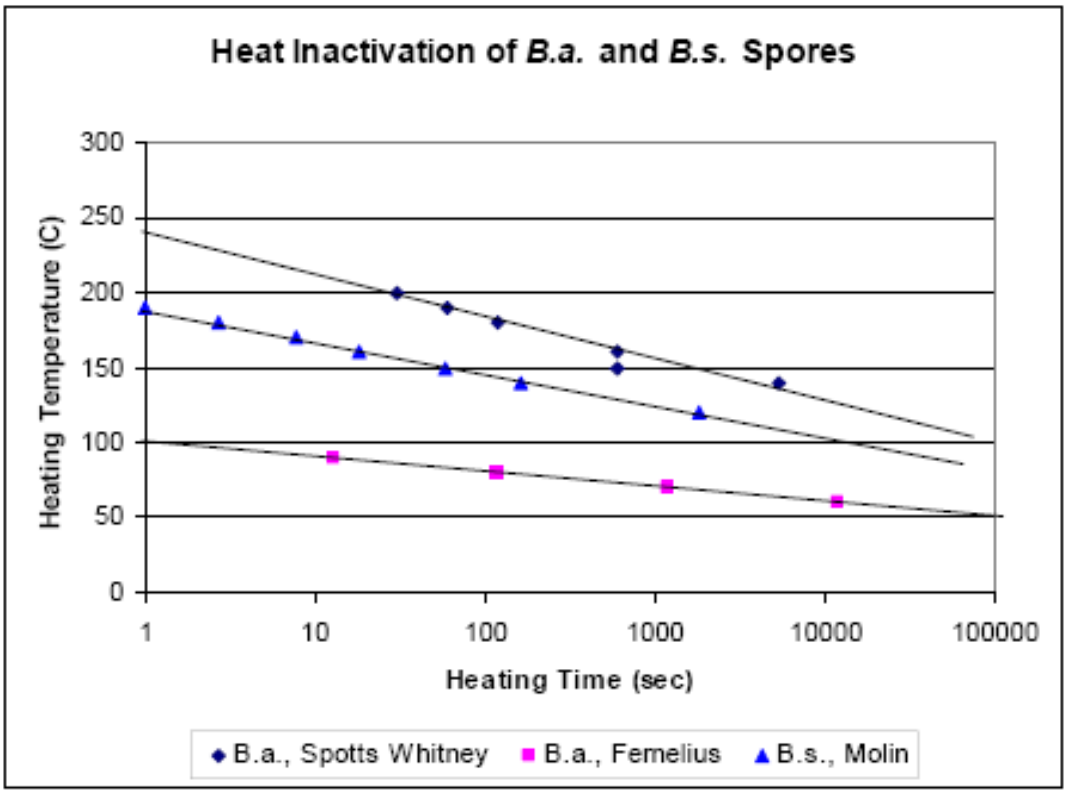

Figure 5. Plot of Dry Heat Inactivation Data for *B.a.* and *B.s.* from three different sources. The diamonds represent the dry heat inactivation of *B.a.* recorded by Spotts Whitney et al as shown in Table 1. The squares are the 99% inactivation for *B.a.* from the Fernelius et al model. The triangles are the dry heat inactivation data for *B.s.* reported by Molin and Ostlund. (Goetz, 2005:23)

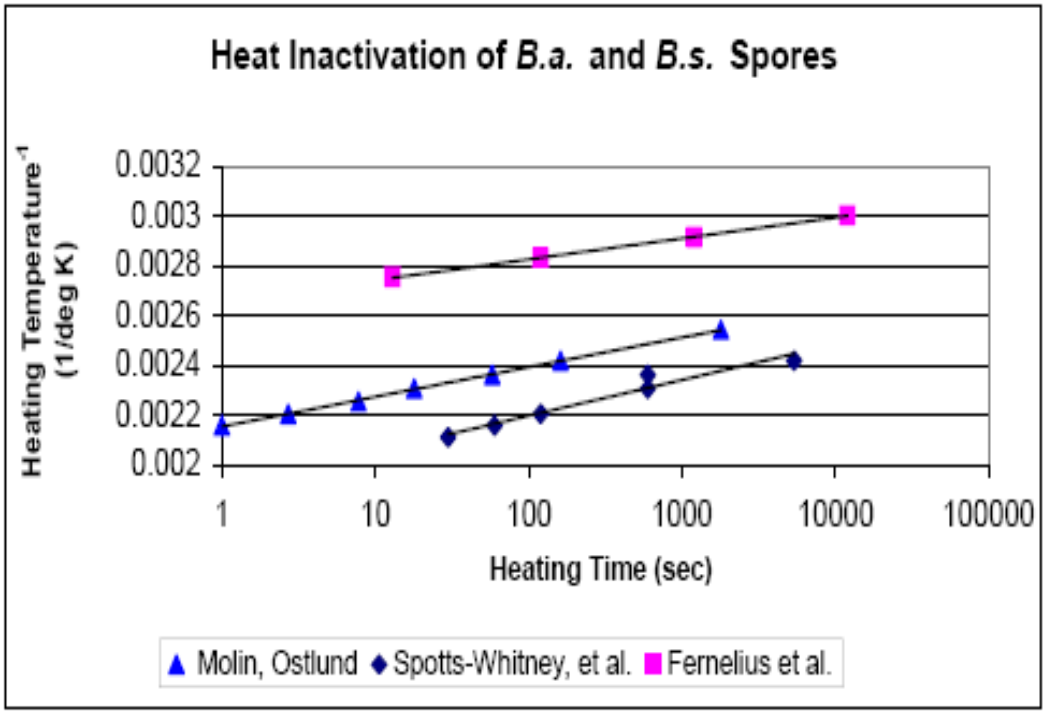

Figure 6. Plot of Dry Heat Inactivation Data Plotted in Arrhenius Style using the same source data found in Figure 4 above. (Goetz, 2005:23)

Figure 6 shows the Arrhenius-style plot of the same data displayed in Figure 5 (Goetz, 2005:23) as predicted. The Arrhenius plot also shows a linear relationship between log heating time and inverse heating temperature. It is evident that temperature exposures for these long times do support the idea of a thermally activated Arrhenius rate reaction within the spores having a constant activation energy over three orders of magnitude of heating times.

III. Methodology

Overview

This research involved the development of a reliable method to create micro-etched standard glass cover slips that would separate *Bacillus* spores enough to aid in germination and growth measurements. This section is a compilation of tested and successful techniques to achieve that goal. Exact heating experimental set up for growth experiments using these platforms are also discussed in detail.

Preparation of Glass Cover Slips

The slides chosen to hold the *B.a*. spores are Gold Seal Cover Glass. These silicate glass rectangles are normally used as cover slips over the larger glass microscope slides. They are 22 millimeters wide and 40 millimeters long with a thickness between .13 and .17 millimeters. They were chosen for several reasons. First, their thinness brings the spores closer to the heated silicate carbide heat sink thereby reducing temperature gradient differences that would result in the much thicker traditional glass slides. Secondly, their small size was much more compatible with the clean room equipment used to prepare the surface making manufacturing easier. Third, once the sample was inverted onto the agar plate, the extreme thinness of the cover slips made vegetative cells easier to see through the microscope. However, a drawback to their use was their fragility in normal handling. Laboratory safety required the use of gloves while handing the cover slips to prevent cuts. Add to this the difficulty in picking them up with forceps, and breakage occurred often, especially around the edges.

The method used to create a specialized sample slide capable of separating and holding spores was wet chemical etching. This procedure entailed several steps in order to prepare the glass surface to be etched. Cleanliness of the surface, as well as an even coating of photo resist was paramount to this preparation. With that fact in mind, all the work done to both prepare and etch the cover slips was conducted in the Clean Room located at AFIT. Both the application and removal of the photo resist followed the procedures outlined in the Standard Photolithography and Pre-Metallization Recipes for EENG 717 Laboratory. However, through trial and error in working with the glass, some modifications were made to the time requirements. All such modification involved an increase in suggested exposure times are indicated within this document.

First, both sides of the cover slip were cleaned. To accomplish this, the cover slips were placed on a Solitec Model 5100 Manual Single-head Spinner system made by Wafer Processing Incorporated. The spinner is shown in Figure 7. The cover slips were centered on a chuck that held the cover slip in place with a vacuum during the spin cycles. While spinning at a rotation speed of 4000 RPM, each cover slip was first rinsed with acetone (CH_3COCH_3) followed by a second rinse with methyl alcohol (CH_3OH). They were then blow dried with nitrogen gas. Finally, each cover slip was placed on a hot plate set at 110 º Celsius for 2 minutes. This last drying step ensured the complete evaporation of any remaining solvents. This cleaning process was repeated again for the remaining side. At this point, the side of the cover slip that was cleaned last was identified as the side that would be etched.

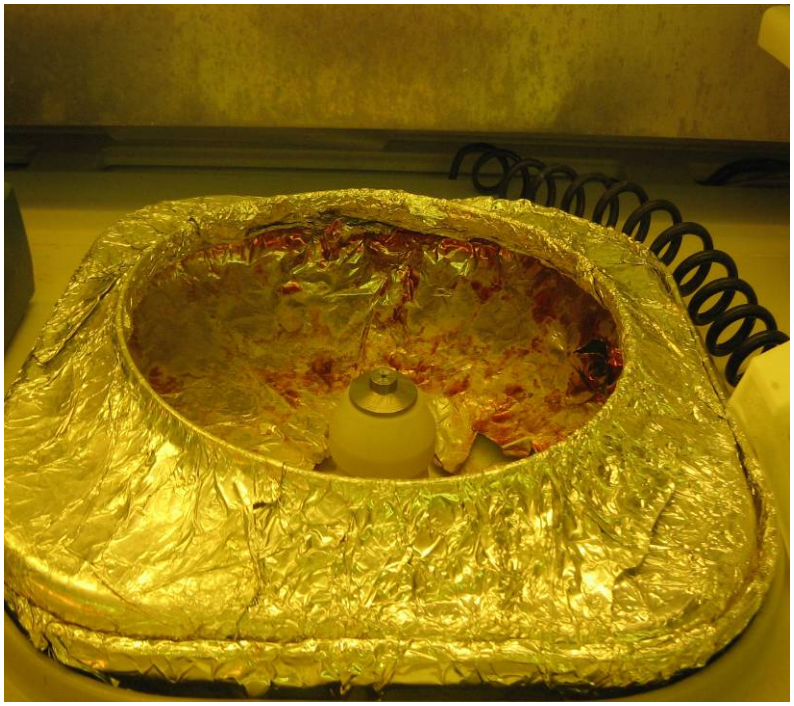

Figure 7. Solitec model 5100 Manual Single-head Spinner. The large vacuum chuck in the center holds the glass cover slip firmly in place while the chuck rotates at the selected RPM. The spinner well is covered in aluminum foil for ease of cleaning as excess photo resist is deliberately flung off the spinning surface by centripetal force. A small drain below the chuck allows excess photo resist to flow into a collection container for proper waste disposal.

Each cover slip was assigned a number. This number was manually etched with a diamond stylus on the back corner. Through every step of the cover slip's lifetime it was tracked using that number. As a result of experimentation, the age of the photo resist mixture as well as its accumulated time on the cover slips surface prior to development proved to be critical. The photo resist tended to harden over time. Its hardness directly effected how long the photo resist needed to be exposed to ultra violet light during the development stage. The full details of the development process are addressed later in this section. Since each step was recorded by date and time for each numbered cover slip,

once this photo resist issue was discovered it was easily identified and adjustments were made accordingly.

The last step in the preparation process was the applications of the photo resist itself. The cover slips received a coating of Rohm and Haas 1818 photo resist. The cover slip was again placed on the spinner and held in place on the chuck with a vacuum seal. Photo resist was dropped on to the center of the cover slip with an eyedropper. Enough photo resist was used to completely cover are area approximately 1.5 centimeters square. In order to ensure an even coat across the entire cover slip, the spinner was activated again to 4000 RPM. The spinner has a ramp up time of a second before it achieved the designated speed. Then the spinner held the cover slip at that revolution speed for 30 seconds. After completing this coating step, the cover slips were again placed on a hot plate to soft bake at 110 º Celsius for 75 seconds. At this point the cover slips were ready for exposure through the mask.

Mask Development

In order to create the divoted cover slip needed to support this method of *Bacillus* spore heating, a material chemical etching technique was used. This technique involved several steps. The first step was to design and create an ultraviolet light mask. The mask was designed to transmit light through a geometric pattern of 3 by 3 micron squares. The squares were set in a grid formation. The entire grid area permitted 100 rows and 125 columns therefore making 12,500 divots per cover slip. The columns were separated by a distance of 500 microns. Rows were separated by a distance of 250 microns. An illustration of the mask is shown below in Figure 8.

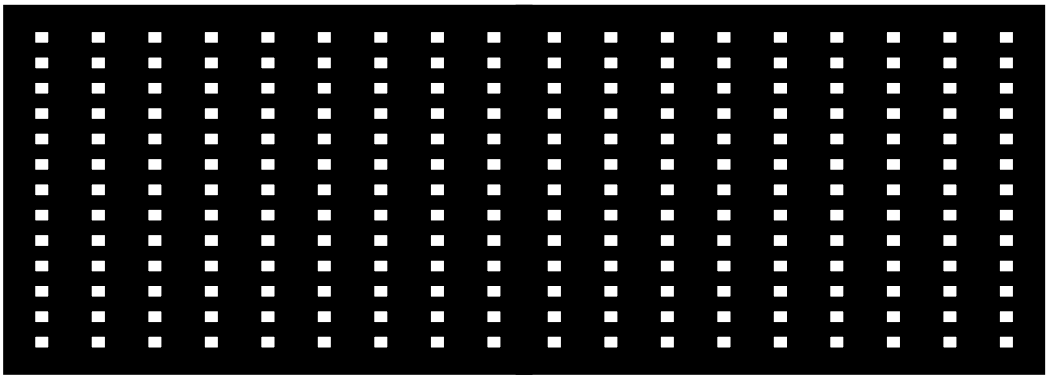

Figure 8. Illustration of Mask Design. The mask was designed to separate the holes at set intervals. The black area represents the part of the mask that was coated with metal which prevented blocked the UV light from reaching the photo resisted surface. The white squares are the 3x3 micron areas where no metal coating was placed outlining the area where the photo resist was damaged by the light during exposure.

The mask was then placed on a Karl Suss MEMS, Micro-Electro-Mechanical System, shown in Figure 9. The photo resist prepared cover slip was mounted directly under the mask platform. In this configuration the mask and the prepared cover slip made physical contact. The cover slip was then exposed through the mask to ultraviolet light for 30 seconds. The light is reflected away from the cover slip by the metal coating on the mask while the light travels through the non-metal coated 3 x 3 micron squares. The ultraviolet light that gets through then reacted with the photo resist coating on the prepared cover slip. Because of the wavelength of the light, the sharp corners of the squares were not exposed by the light waves so the resulting exposure area took on a rounded shape instead. Figure 10 shows photo resist covered cover slip with “circular’ exposure.

Figure 9. Karl Suss MEMS. The mask aligner has a movable stage where both the mask and the cover slip platform are inserted under the UV lamp. This stage is adjustable in both the horizontal and vertical directions. The eye piece and camera allows the user to manually align the mask and platform surface horizontally. In the vertical plane, it is critical for the mask to make mechanical contact with the photo resisted surface to prevent the UV light from damaging the area surrounding the mask windows.

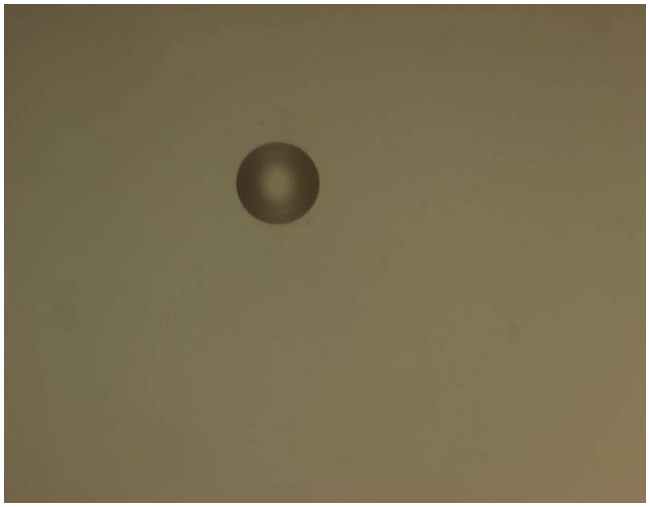

Figure 10. Magnification at 400x of exposed photo resist. Although the mask is designed with square windows, the wavelength of the UV light is so long that it does not fit in the corners of the squares and therefore the resulting damage to the photo resist takes on a circular pattern. Here is a picture of that circular damage.

The next step in the etching process is the removal of the photo resist areas on the prepared cover slip that were damaged by the ultraviolet light exposure. These cover slips were submerged in Microposit 351 developer solution. This developer consists of 5% sodium hydroxide, 10% sodium tetraborate decahydrate, and 1% inorganic borates diluted with water. The developer was mixed with deionized water at a ratio of 1:5. Typical developer time for damaged photo resist removal is usually around 30 seconds. However, over the course of several development attempts it was discovered that exposure time to the 351 developer needed to be at least 2 minutes. This is possibly due to the extremely small areas of the 3 x 3 micron "circles".

Once the photo resist was removed in these areas the actual etching step was next. In order to protect the even surface needed for heat conduction the back of the cover slip had to be protected from the etching solution. To accomplish this, the cover slip was mounted to a regular glass microscope slide. It was affixed to the sacrifice slide with additional photo resist. This technique effectively kept the etching acid from reaching the back of the cover slips and causing the aforementioned damage. Below is picture of this process.

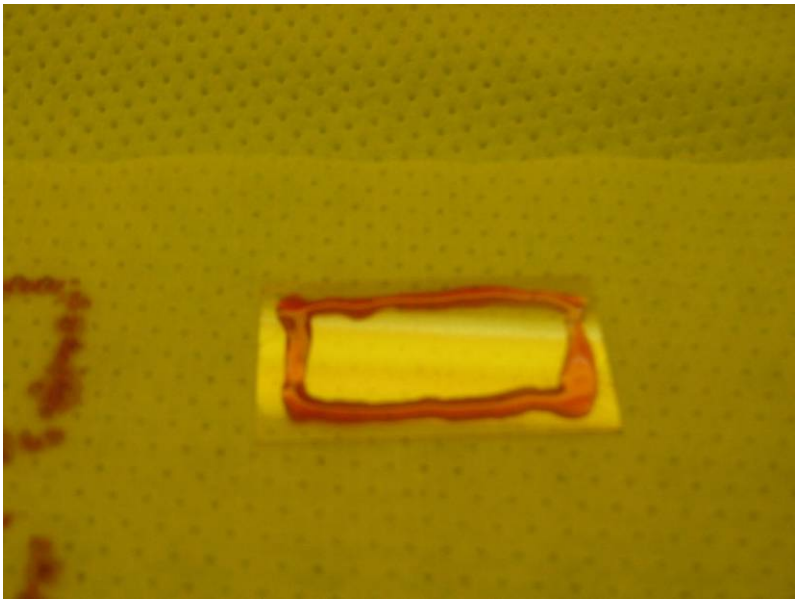

Figure 11. Inverted exposed cover slip with photo resist "glue" surrounding edges. The exposed cover slip is inverted with more photo resist applied around the edges for a duel purpose. First, this additional photo resist acts like a glue that will adhere the cover slip to a sacrifice slide. Second, the photo resist is applied to the edges to create a barrier that keeps the BOE from etching the underside of the cover slip.

After the sacrifice slides were prepared, the cover slips were ready for the acid etching step. The fundamental reaction in the etching process is:

$$SiO_2 + 6HF \rightarrow H_2SiF_6 + 2H_2O.$$

The cover slips were immersed in buffered oxide etch solution (BOE). The BOE solution is a made with six parts of 40% NH_4F diluted with water mixed with one part 49% HF diluted with water. BOE was chosen over straight hydrofluoric acid since the solution allowed for a slower and therefore more controllable etch rate. The BOE etched the glass cover slips where the photo resist was removed by the developer. The glass remained intact and protected from the acid where the photo resist remained. Cover slips were etched for 10, 15, and 20 minutes to determine both the etch rate and optimum hole size needed for spore capture.

Finally, the photo resist was removed. Since the cover slips were essentially glued to the sacrifice slides, the slides needed to be soaked in acetone for more than 12 minutes before the photo resist was dissolved enough to release the cover slips. Additional soaking time was then needed to remove whole of the underside photo resist/glue. Once the visible photo resist film was removed, the cover slips were wiped clean with more acetone followed by methanol. The final result was the glass cover slips with divots or holes etched in the grid pattern matching the original mask design.

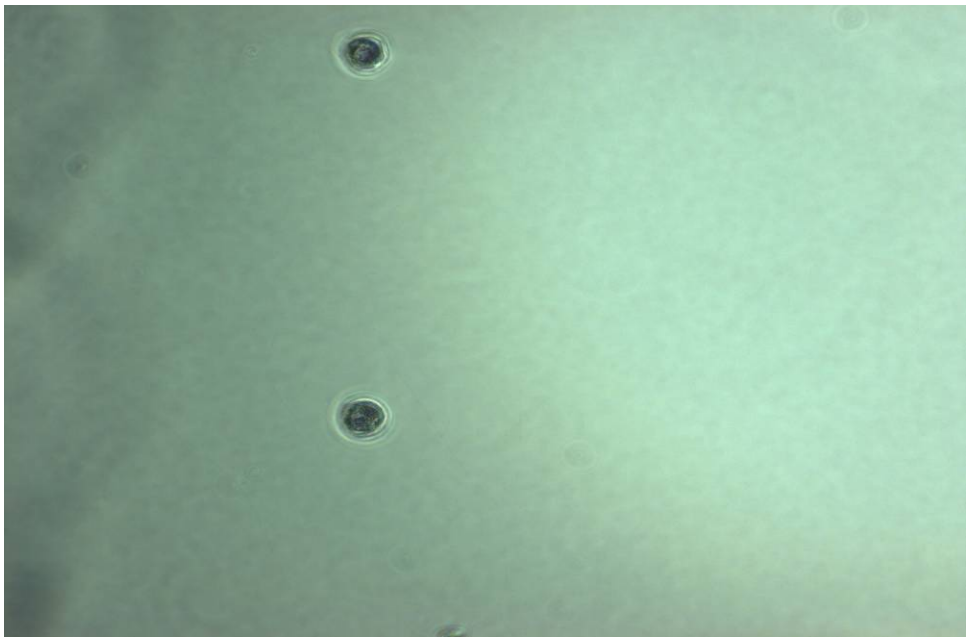

Figure 12. Two holes at 200x magnification. These two holes are in a column. The surface displays signs of the etching process as a series of concentric rings. It is impossible to determine the depth profile of the hole from visual inspection with the light microscope.

SEM Verification

After the holes were etched, a Scanning Electron Microscope (SEM) was used to view the holes. Since the SEM requires a chargeable surface to create an image, a conductive coating was placed on to the etched platform's surface. Gold was selected because of its conductivity as the coating element, as well as its ease in its layered preparation using an evaporation technique. In order to prevent the surface from building up too high a charge which would obscure the SEM image, a 25 Angstrom coating of gold was applied to the surface.

This was a very revealing step in the platform development process. First, it provided a method to measure the holes themselves. This confirmed the published etch rate for the BOE on glass. Second, it illustrated the physical evolution of the etch process. The surface did not experience a uniformed etch. Unlike a hole that would be drilled straight down that would result in a hole that resembles a cup, the BOE solution essentially puddled and etched the center of each hole dramatically while only gradually etching the edges. The resulting hole shape resembled a funnel much more closely than a cup or a bowl. Figure 13 below shows this resulting shape.

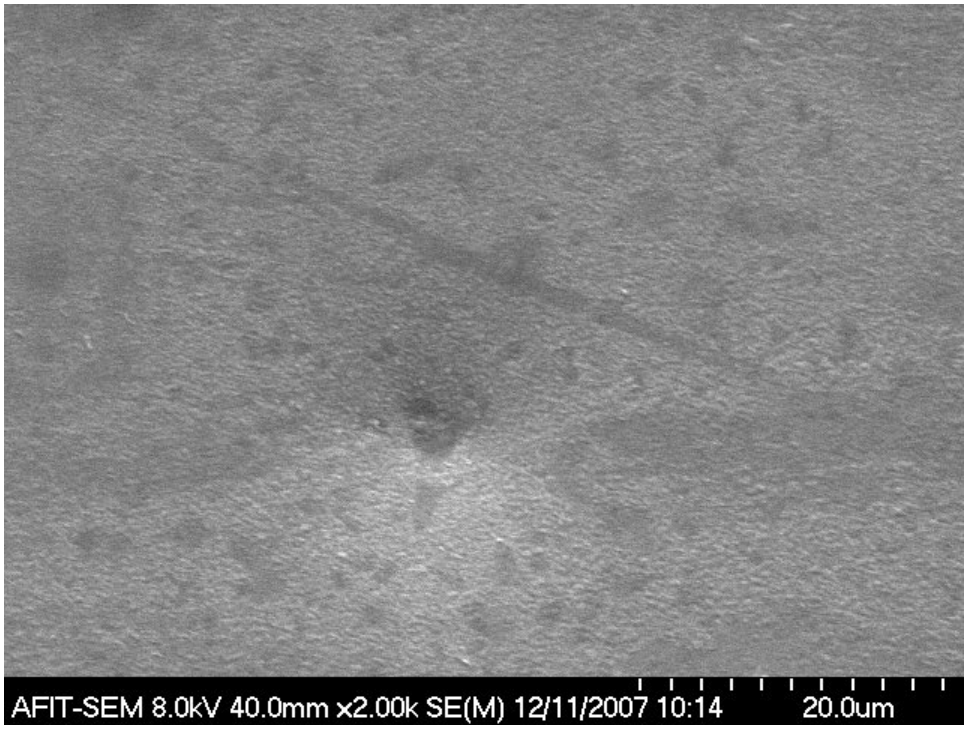

Figure 13. SEM Image of a hole. The scanning electron microscope was used to determine the depth profile of the holes. From this view, the shape of the hole is less like a cup and more like a funnel where the hole is a gradual slope at the top and a dramatic sink at the center point.

Spore Preparation

Both the samples of *B.t.* and *B.a.* used in this research were obtained from Dr. Eric Holwitt of the Air Force Research Laboratories, Biomechanisms and Modeling Branch, Brooks City Base, Texas. The samples were stored in the form of lyophilized spores and kept in glass culture tubes at room temperature.

The spores were spread on nutrient agar plates. They were incubated at 34 º Celsius for 48 hours. The next step was to harvest those spores. Sterile water was pipetted onto the culture plate covering the plate surface. Next the spores were mixed with the water using a spread rod until a thick film was visible above the agar base. The resulting solution was pipetted into a centrifuge tube.

Spore Slurry Preparation

In order to clean up the spores and remove the debris, the mixture was centifuged at 4000 revolution per minute for twenty minutes. At the end of this cycle the spores settled out at the bottom of the tube while the supernat rose to the top. The excess liquid was drained and more sterile water was pipetted in to return the tube to 6 ml. A vortex mixer was the used to break up the spore pellet and re-suspend the spores in the water. This cycle was repeated three times. After the last spin cycle the water was not replaced and the pellet of spores at the bottom of the tube was not re-suspended. These cleaned spores were then stored in the refrigerator at 4 º Celsius until use, see Figure 14 below.

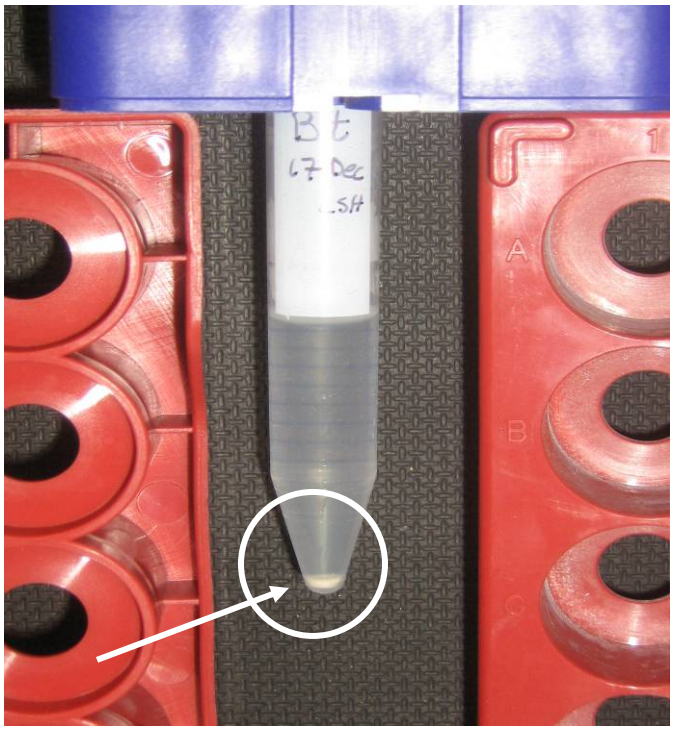

Figure 14. Spore pellet located at the bottom of the centrifuge tube. Spores were harvested from an agar plate and then placed in a centrifuge tube for cleaning. After each spin cycle the supernat was poured from the tube and refilled with sterile water. This rinse and spin was repeated three times to remove cellular debris. The final, cleaned spore pellet settled at the bottom of the tube.

Application of Spores to the Cover Slip

The major hurdle of the project required developing a technique to get the *Bacillus* spores into these manufactured holes. The problem was simply a ratio issue. The amount of area on the cover slip that was a hole compared to the amount of surface area that was flat is less than 1%. To that end, the spore solution had to remain at a very high concentration. Finding the best method to accomplish this took several attempts. Brief discussions of these attempts are included in the Appendix. The final application technique addressed both the problem of putting spores down on the cover slip and getting a spore into a hole as well as the second problem of removing the remaining spores that were not in the holes off of the surface.

The surface of the cover slips was first coated with a detergent wash. Commercially available Ivory soap shavings were diluted with sterile Millipore® water in a suspension of 2 grams soap to 250 ml water. The detergent solution was then sterilized in the autoclave at 121 º Celsius at 15 psi for 15 minutes. The detergent solution was placed on the cover slips using a sterile pipette. The solution was then rinsed off with pipetted, sterilized water. The purpose of this was two fold. First, any remaining detergent served to reduce the surface tension of the water in the spore solution, permitting easier access to the etched holes. Second, it helped to coat the cover slip surface to provide an additional layer that aided in spore removal that will be addressed in the following passages.

The cleaned but undiluted spore slurry was pipetted directly from the centrifuge tube and dropped on the cover-slip. Next the slurry was spread using a sterile wooden tooth pick over all the holes. Any water on the spore laden cover slips was then allowed to evaporate. Since *Bacillus* spores are not motile, the evaporation of the water forced

the spores to settle directly on the cover slip surface. Figure 15 below, is a representation of these steps. Next, in Figure 16 are pictures that show that the spore slurry employed by this method was thick enough to indeed cover the surface.

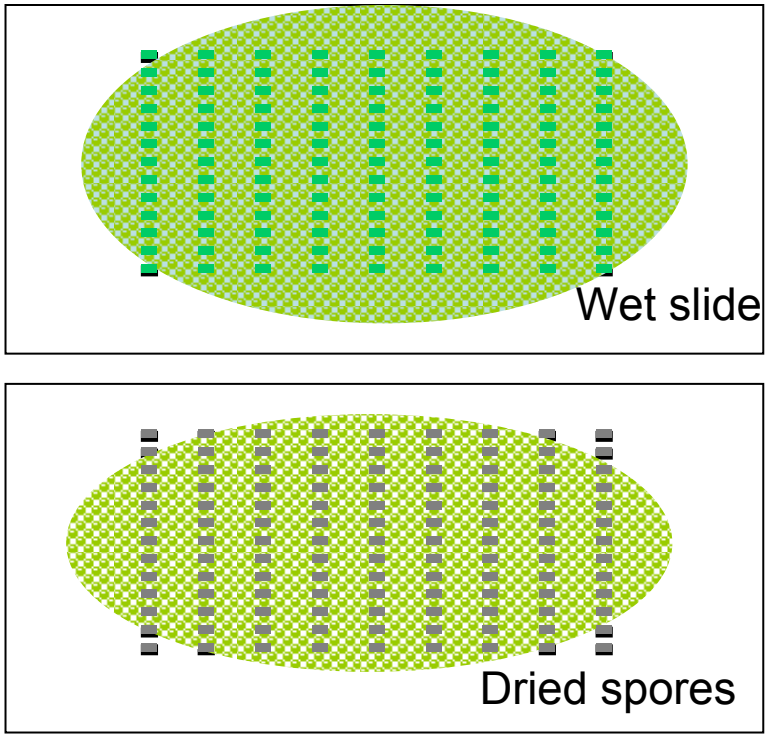

Figure 15. Illustration of Spore Application over holes. The wet spore slurry was undiluted when it was pipette into a large droplet that covered the etched hole grid. The water then evaporated and the spores were allowed to settle directly on to the slide surface. The dried spores appeared as an opaque film covering the slide surface.

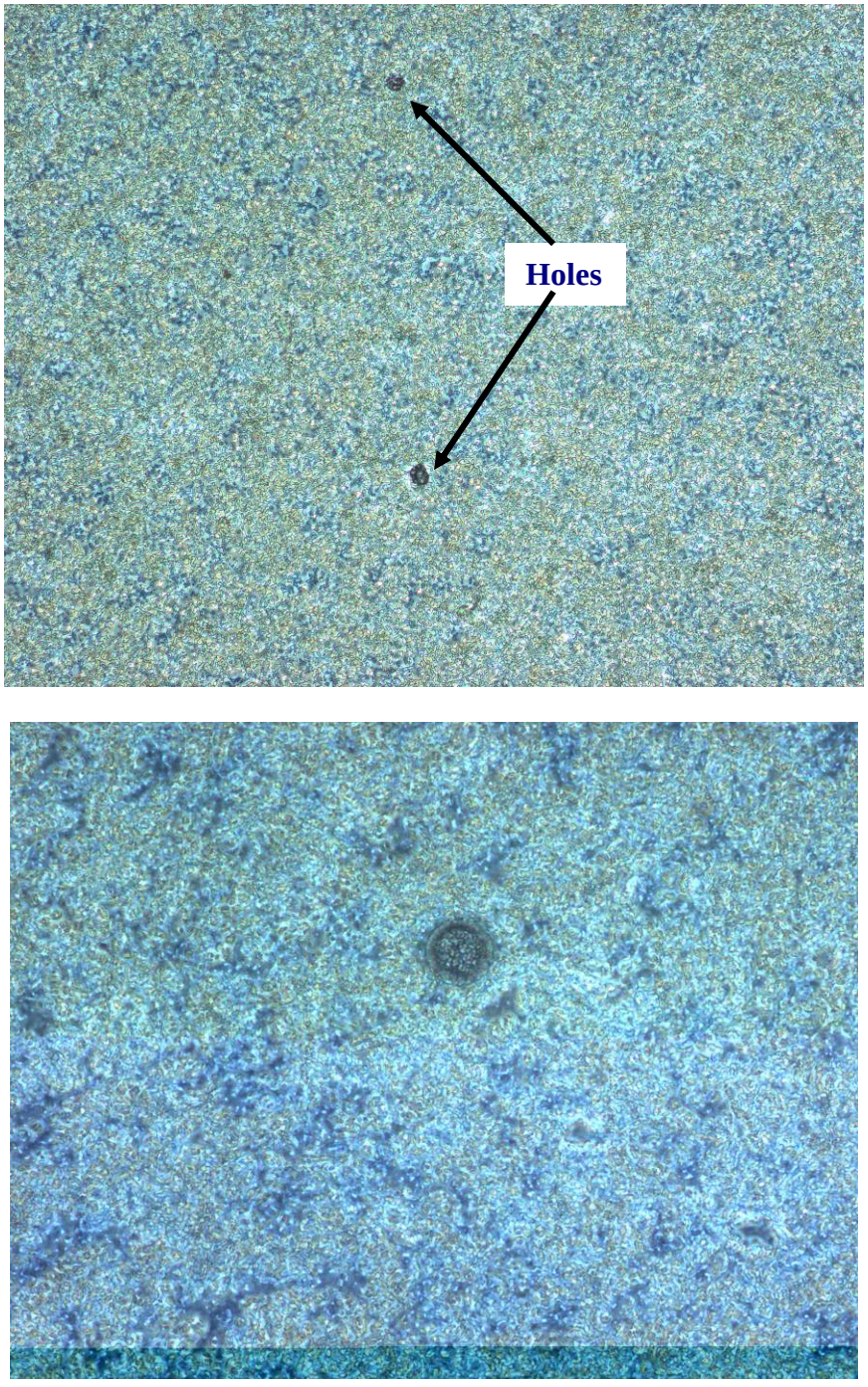

Figure 16. Cover slip with dried spores covering holes. The pictures were taken at 200x and 400x magnification respectively. The high concentration of spores in the slurry improves the probability that a spore will find a hole on the surface. However, even at this high magnification it is difficult to visually confirm that there are spores in the holes.

Cleaning all of the extra spores off the surface was the next step in sample preparation. The trick to this process was to remove the surface spores while avoiding disturbing those spores already in the holes. This also took several attempts to solve and methods are also discussed in the Appendix. The final technique used was mechanical, while avoiding marring the glass surface.

The spores were sheared off of the surface using successive wipes with both a plastic edge and a paper one. Plastic was chosen since the glass surface of the cover slip was prone to scratching which hindered spore removal efforts. The plastic edge was made by cutting a laboratory weight boat into approximately 3 inch squares. The squares were folded over for strength and the manufactured edge was used to shear the spores off the surface. This took several wipes. Once the gross removal of spores was accomplished, the succeeding wipes were made with folded weigh paper. Weigh paper is non-absorbent, preventing potential removal of spores by water molecule attraction from the holes. The cover slips were visually inspected by light microscopy at multiple intervals for surface cleanliness.

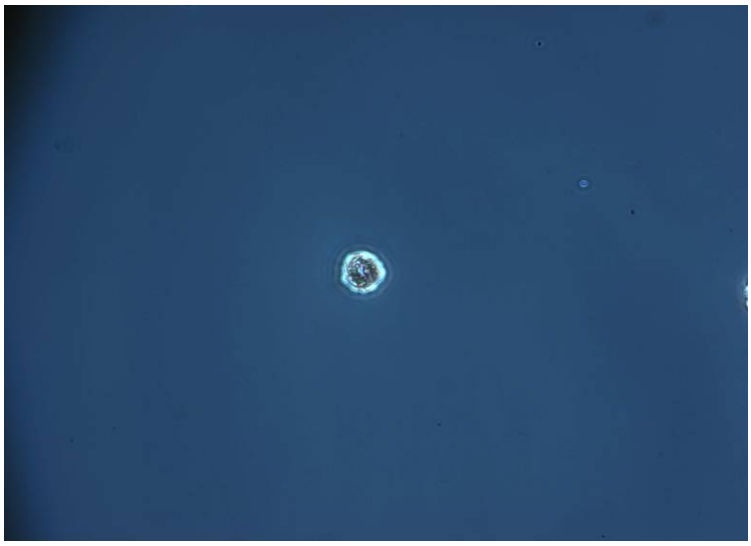

Figure 17. 400x magnification of Cover slip after the surface has been cleaned with successive mechanical shearing. The surface has been successfully. Note the presence of spores in the hole is still difficult to visually confirm.

Agar Preparation

The spores were grown on nutrient agar. The agar used for this research was manufactured by Criterion. This is a standard recipe for growing bacteria. The approximate formula is 15.0 gm agar, 5.0 gelatin peptone, and 3.0 gm beef extract. The nutrient agar consists of common ingredients and stored as a powder. The inclusion of a beef extract provides salts, vitamins, nitrogen, and carbohydrates the bacteria uses for growth activities. A peptide is added to control pH. The agar is a solidifying ingredient made from red seaweed. The powder is prepared with heated distilled water and, once combined, is poured into sterile petri dishes.

Normally, once the agar is poured into the petri dishes and reaches room temperature it is ready for use. For this application it was discovered the water content of the agar was too high. The water that allows the nutrients to move freely among growing

colonies also allowed the germinating *Bacillus* spores to move out of their holes and travel across the clean surface. To alleviate this problem, the agar was allowed to dry out at room temperature for 36 hours before it was deemed ready for use as a growth plate.

Heating in the Furnace

A standard laboratory ceramic box furnace was used to expose the sample of spores on the cover slip. The furnace was a Vulcan Box Furnace Model 3-130 with programmable controls. It has a temperature range of 50 º to 1100 º Celsius with 1º resolution. This furnace was operated at a steady state temperature where it has a published temperature accuracy of ± 5 º Celsius. The temperature uniformity throughout the oven chamber during steady state operations is ± 8 º Celsius.

This accuracy range was too large for the purposes of this experiment, so additional modifications were made to the furnace chamber prior its use. First, a smooth, 3 inch diameter puck made of silicon nitride (Si_3N_4) was centered in the base of the chamber. Silicon nitride was developed in the 1960s as a replacement material for metal engine parts with ceramics to allow higher system operating temperatures and efficiencies. Its hardiness and ability to hold high temperatures without loss of structural integrity made this material perfect for use as a heat platform within the furnace. Since uniform heating was important, this heated platform ensured an even, steady heat distribution to the cover slip while the furnace door was lifted to emplace or remove it. See Figure 18 below.

Figure 18. Vulcan Ceramic Furnace Oven Chamber. The oven door is hinged at the top which adds additional time to manipulate samples both into and out of the chamber. The furnace can be set to run at a steady state temperature or adjust the chamber temperature at regulated intervals.

A second addition to the furnace set up was a thermocouple. The thermocouple wire was affixed directly to the puck surface to measure the temperature of the surface coming in contact with the sample cover slip. The thermocouple compares the thermal gradient of two different types of wires to convert thermal potential to electric potential. The relationship between voltage and temperature can be approximated by the following equation;

$$Temperature = \sum_{n=0}^{N} a_n * (output\_voltage)^n \qquad \text{(Equation 7)}$$

where a_n is the material coefficient. The thermocouple used for this was a type K, the most commonly used general purpose variety. The wires used in this type were both nickel alloys. One wire was made of chromel (90% nickel and 10% chromium) while the other one was alumel (95% nickel, 2% manganese, 2% aluminum, and 1% silicon). The measurable temperature range of the type K is from 0 º to 1100 º Celsius. The accuracy tolerance however varies with temperature. For most of the experiments which are between 0 º and 375 º Celsius the accuracy is ± 1.5 º Celsius. However, at temperatures above 375 º Celsius the accuracy changes proportionally to the temperature with the formula:

$$Tolerance = \pm 0.004 * Temperature(Celsius) \qquad \text{(Equation 8)}$$

The thermocouple wires were held mechanically in contact with the puck surface using copper mesh tape and steel washers. The thermocouple was attached to FLUKE 298 multimeter which was operating in temperature mode.

The sample cover slips were both placed on the puck in the furnace chamber and removed manually using two metal spatulas. After repeated practice trials with a 500 degree Celsius chamber temperature, lifting the chamber door and placing the sample on the puck took 3 seconds ± 1 second. Removing the sample cover slip after it was heated took longer with an average of 4 seconds ± 2 seconds which also includes the lifting of the chamber door. This made the thermocouple reading important. Since the temperature reading of the box furnace is of the whole heating chamber, when the oven door is lifted the temperature reading would dip 10 ± 5 º Celsius and take 30 ± 10

seconds to return to the chosen steady state temperature. However, since the interest was the surface temperature of the puck, this is reading the thermocouple provided. After the chamber door was lifted the temperature reading on the thermocouple would drop 3 ± 1 degree Celsius, but this would not happen immediately, unlike the oven measurement. The recovery time back to the designated steady state temperature was 5 ± 0.5 seconds which is the cycle reading time built into the multimeter.

The thermal conductivity across the cover slip is determined using the Fourier's Law for heat transfer by conduction.

$$Q = k * A * \frac{T_2 - T_1}{x} \quad \text{(Equation 9)}$$

where

- k is the thermal conductivity of the material (Watt/(m* ºC))
- A is the surface area (m^2)
- x is the distance through the material (m)
- T_1 and T_2 are the surface temperatures (ºC)

This equation was used to determine the heat transfer through the glass cover slip from the puck surface to the spores. Below is Equation 9 with the variable values for the glass and the cover slip dimensions. The temperatures used were the highest furnace temperature of 180 ºC and room temperature of 25 ºC.

$$Q = (\frac{0.8 Watts}{m * °C}) \times \left[\left(22 \times 10^{-3} m \right) \times \left(40 \times 10^{-3} m \right) \right] \times \left(\frac{(180 - 25)°C}{0.17 \times 10^{-3} m} \right)$$

$$Q = 642 Watts$$

The time of this heat transfer through the glass cover slip is therefore fast enough to consider it negligible for the purposed of temperature error.

Initial Furnace Trials with Scratched Cover Slips

In order to narrow the experimental temperature range, preliminary tests were run to determine possible time and temperature threshold points. These tests, using the furnace heating procedures, recorded only if there was spore germination. For these preliminary tests, in the interest of ease and material conservation, differently designed cover slips were used to hold the spore samples.

The cover slips holding the spores were the same Gold Seal ones described earlier; however, instead of the etching holes, silicon carbide sandpaper was used to scratch grooves into the surface. Although the grooves were in irregular depths and patterns on these cover slips, the intent was to catch spores in these grooves and simulate the etched slides where the spores would also be located slightly below the glass surface. Since this arrangement does not lend itself easily to statistical analysis, the results of these tests were determined using only growth, any growth as the benchmark event. The spore solution was deposited on the cover slip in the same way described with the etched cover slip. The spore solution was allowed to dry and the surface rinsed and wiped clean again as described before. These sample cover slips were placed in the box furnace and heated at set intervals. Figures 19 show these scratched cover slips.

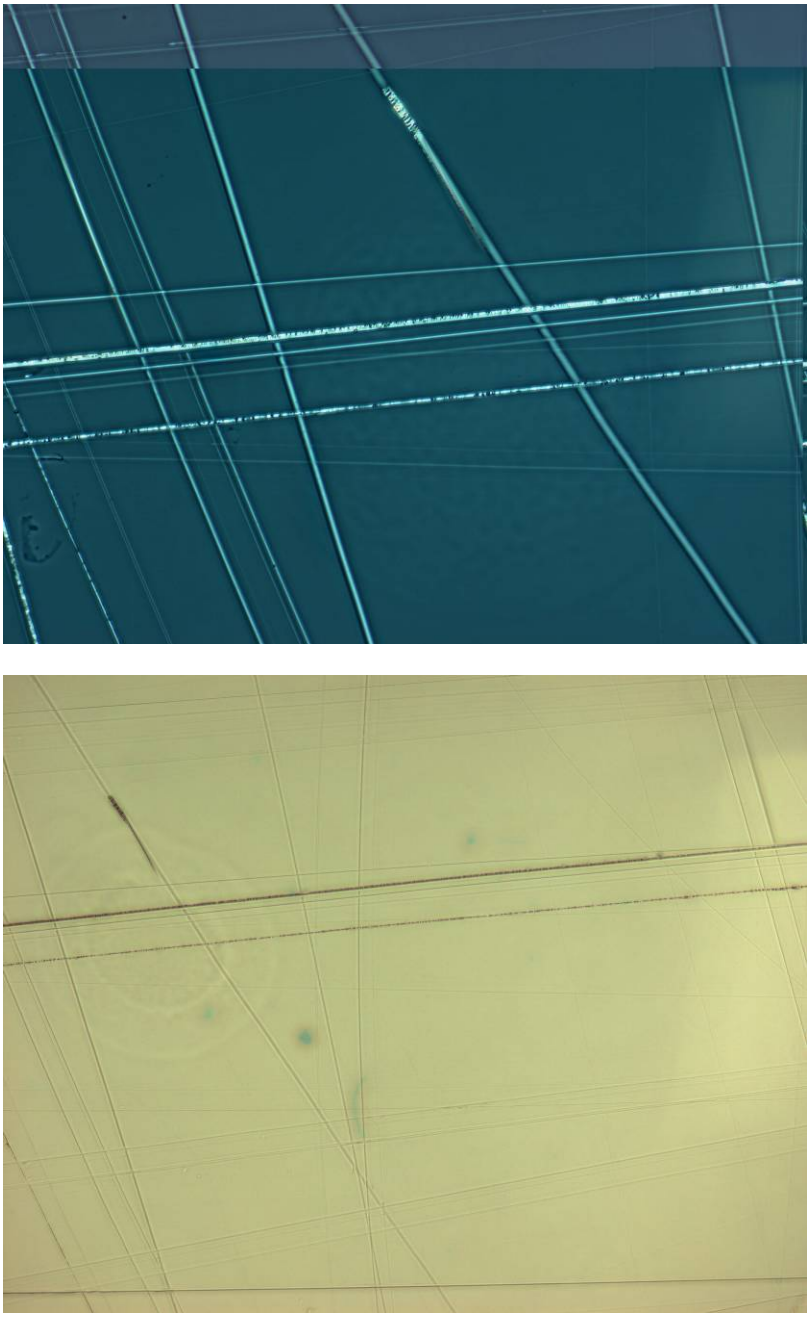

Figure 19. Scratched slides with spores. The silicon oxide sandpaper scratched the surface in a varied pattern. The darker areas in the scratches were visual evidence of spores being caught in the grooves. With this method the spores are spread irregularly throughout the scratched area and it was impossible to determine the amount of total spores present.

IV. Results and Analysis

Chapter Overview

This chapter contains the results of the experiments using methods described in the previous chapter, providing both numerical and visual results of analysis for timed heat experiments of *Bacillus anthracis* and *Bacillus thuringiensis*. The first set of experiments dealt solely with the task of growing spores in holes. Various methods of application and removal were tested before settling on the technique described in the previous section. The measurement of success was determined visually. The criteria were simple. First, the spore growth had to be centered on the holes which indicated that the spores were both in the holes and able to germinate there. Second, since overgrowth was a stated problem, the area between the rows and columns had to be clear of excess spores. Figure 20, below, shows the results of the successful techniques.

Figure 20. Successful Growth in Holes. This is the culmination of multiple attempts of both spore application and removal until a repeatable, reliable technique was developed. The center of gravity for the growth of *B.t.* on this etched slide is clearly the holes. The area between the columns is clear of excess surface spores.

Furnace Trials with Scratched Slides

Overview

The purpose of the initial heating trials using the scratched cover slips was to establish a baseline death response from both the *B.a.* and *B.t.* spores. Since most of the previous research dealt with heat exposure times well beyond one minute, new data had to be collected. Also, as had been mentioned earlier, spore preparation and water content greatly influences the death response. No research used this method of non-diluted spore slurry so it was reasonable to expect the death response to differ slightly from predictions using other data. This expectation of a different kill rate was validated with the first set of experiments.

The next goal was to test the theory of using *B.t*. as a surrogate for *B.a*. in heat trials. To this end, both the *B.t*. spores and the *B.a.* spores were stored, grown, prepared, and killed utilizing the exact same methods and environments. This removed controllable variability between the *Bacillus* species and increased the confidence in continued use of such surrogacy.

Heating

Bacillus thuringiensis was selected because of its lower Bio Safety Level classification as the subject of the initial heat trials. The focus of these trials was to both find the related temperature and time point where no spores were able to germinate after exposure. Additionally, the *B.t.* was used to narrow the temperature range for subsequent trials with *B.a*.

The scratched cover slips provided a surrogate for the etched cover slips. The spores seemed to get caught in the grooves, especially at line intersections. The unmarred surface was also able to be cleaned in the proposed manner providing a separation between spores. These characteristics made any vegetative growth easy to find. All slides were checked for growth after 5 hours of incubation at 34 º Celsius. The Figures 21 and 22 below show the spores in the grooves and subsequent growth.

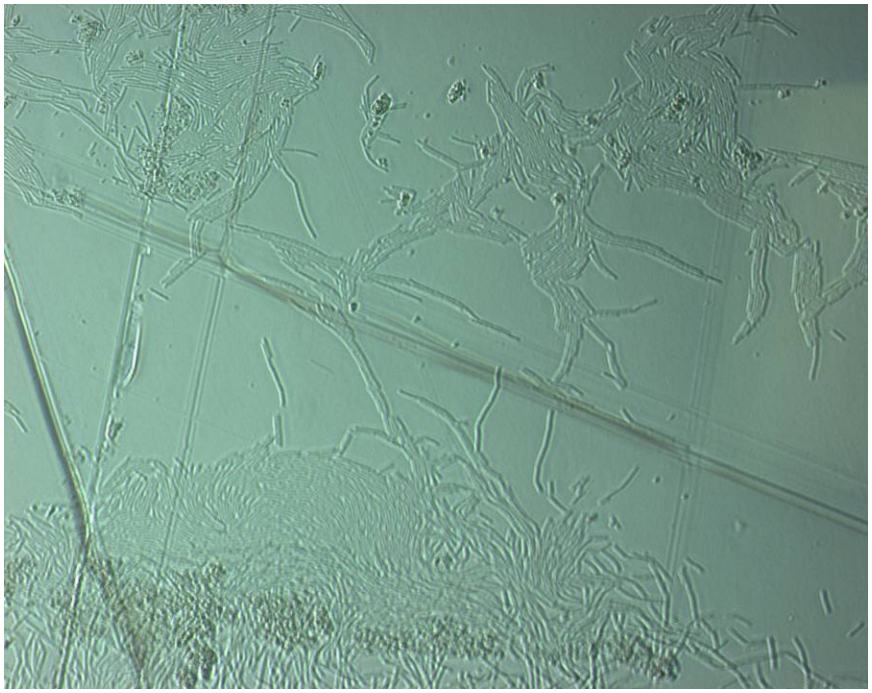

Figure 21. *B.t.* spore growth after 5 hours on scratched slide. The spores tended to cluster at points were the scratched lines intersected. Since vegetative cells grow linearly once the spores germinated, if they were close to a scratch they tended to stay in the groove until their mass or other cells forced them out.

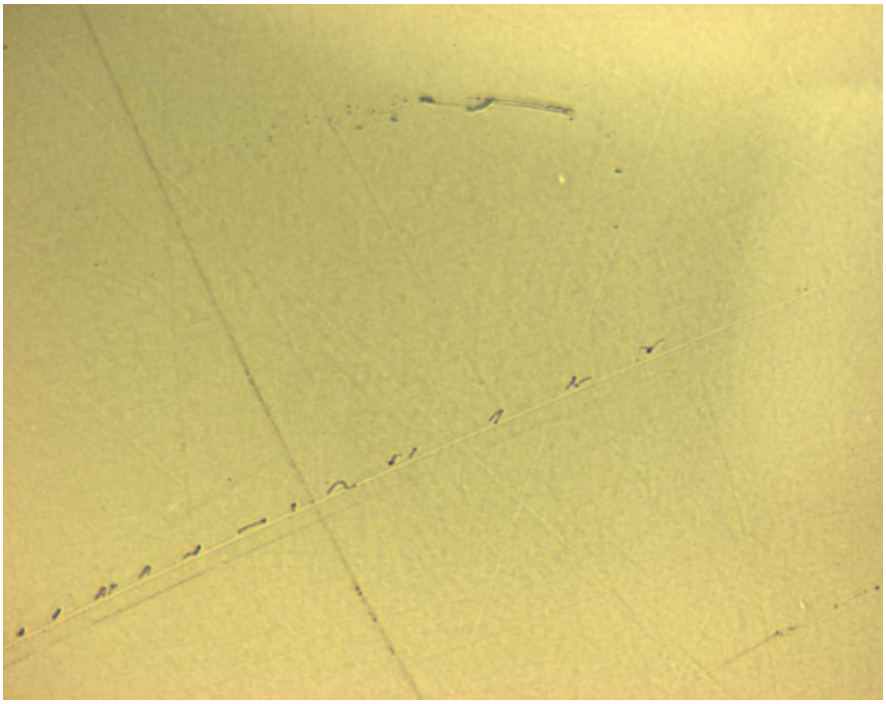

Figure 22. *B.t.* starting growth after 3 hour along scratch. This is the very early germination pattern of spores caught in a scratched grove. As stated in Figure 21 above, the vegetative cells tend to stay in the groove.

The resulting data revealed by these trials for *B.t.* is displayed below in Figure 23.

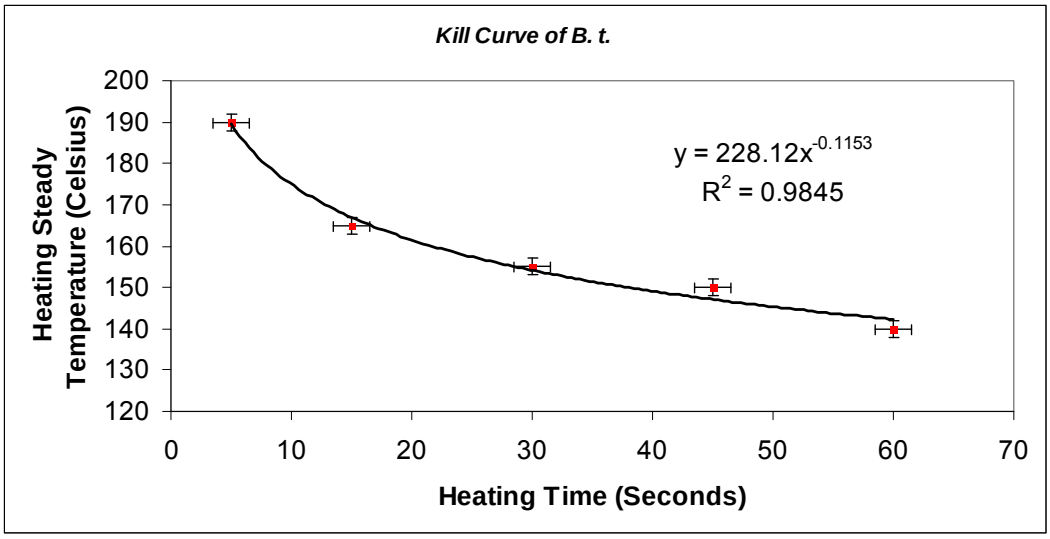

Figure 23. Kill Curve of *B.t*. The *B.t.* spores were heated on the scratched cover slips at a steady state temperature at set time intervals. The kill point was determined to be the timed exposure point where no spores germinated after five hours incubation.

The same heat trial was repeated using *B.a*. and this resulting data is displayed below in Figure 24.

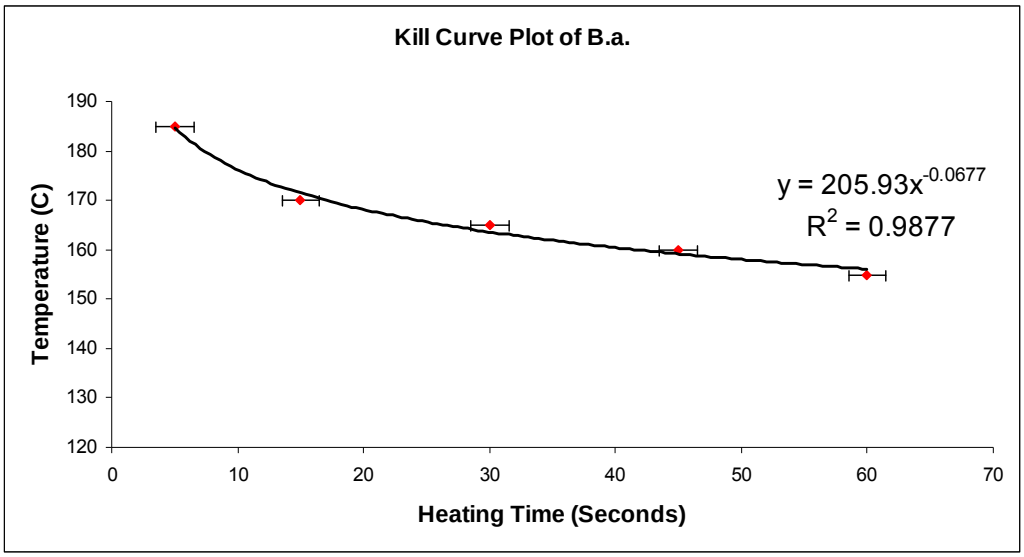

Figure 24. Kill Curve of *B.a.*. The timed exposure at steady temperatures experiment was repeated using *B.a*. spores. The same kill point definition was used.

Looking at both of these *Bacillus* species together in Figure 25 the similarity in heat response is evident. *B.a*. as expected seems to be the more resistant to heat damage of the two, as predicted using other research data. However, the temperature that death occurred was much lower than predicted with published data. I suspect this is related differences in the preparation and storage of the spores.

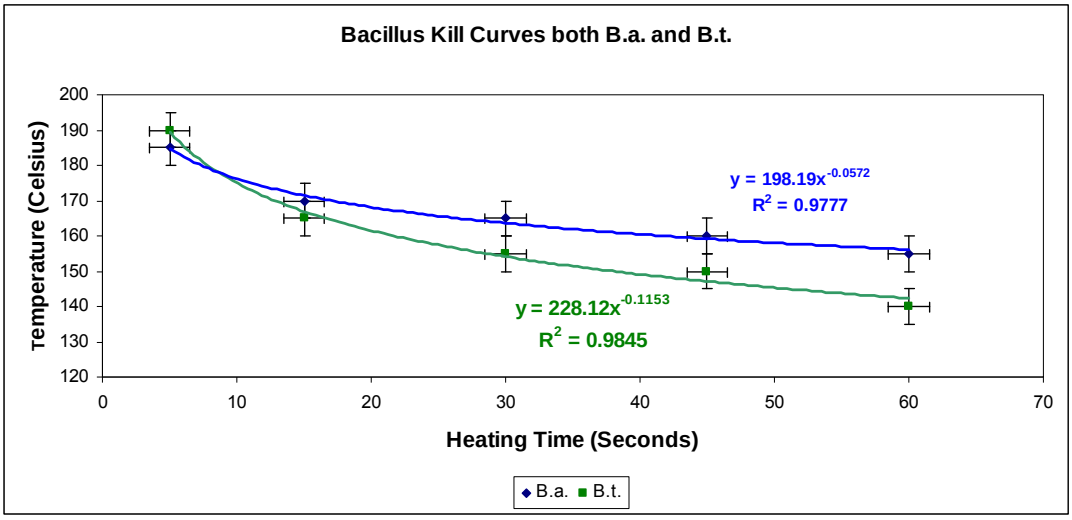

Figure 25. Bacillus Kill Curve of both *B.a.* and *B.t.* Here are the kill curves for both species shown on the same graph. The crossing point of the two curves around the 10 second point was the focus of the next experiments on *B.a.*

An additional question addressed by this test was whether the inactivation response is consistent with the Arrhenius relationship. Figure 26 shows the same data plotted in an Arrhenius format. Both *B.t.* and *B.a.* followed this relationship which supports the repeatability of this experiment.

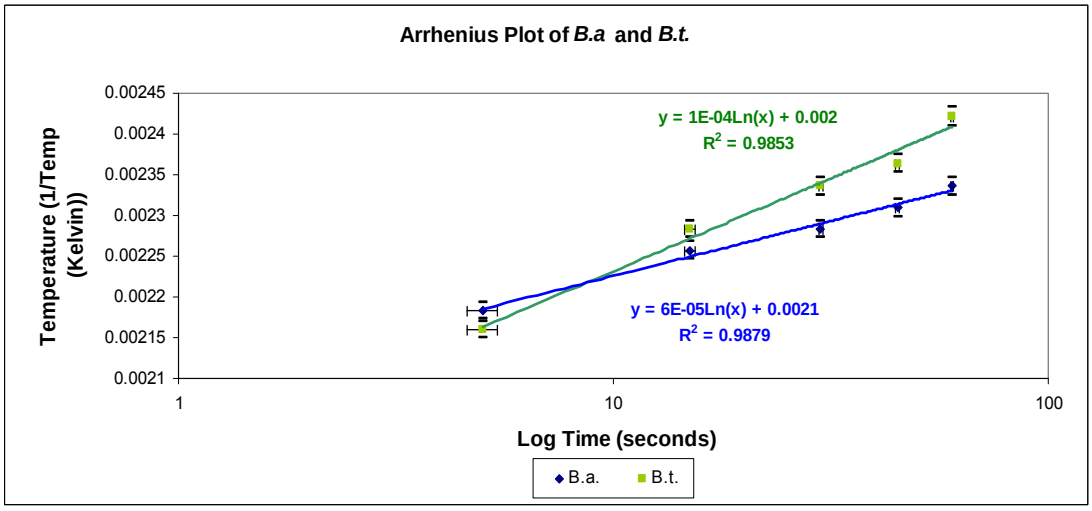

Figure 26. Arrhenius Plot of *B.a.* and *B.t.* Here are the kill curves for both species are plotted using the Arrhenius equation. The data for both species supports the anticipated linear threshold relationship at these time intervals.

The Arrhenius plot relationship shows the value of the slope of the trend-line is $\left(\frac{E_a}{R}\right)$. Using the line equations shown on Figure 26 for *B.a.* and *B.t.* the activation energy for the spores was calculated. The slope of the *B.a* data is 4.15×10^{-4}. The slope of the *B.t* data is 6.9×10^{-4}. By using this slope relationship the activation energy for *B.a* is $E_a = slope \times R = 4.15\times10^{-4}\times8.314 = 3.45\times10^{-3}\left[\frac{Joules}{mol}\right]$. Doing the same for *B.t.* shown an activation energy of $E_a = slope \times R = 6.9\times10^{-4}\times8.314 = 5.74\times10^{-3}\left[\frac{Joules}{mol}\right]$.

Heat Damage and Growth Trial

The purpose of this experiment was to use the micro-etched cover slips capitalizing on their ability to separate vegetative growth to track changes in growth of *Bacillus anthracis* after heat injury. The time of exposure was held constant at 10 seconds. This time was chosen based on the combination kill curves for these organisms as seen in Figure 26 above. The ten second area is close to the point where the two kill curves cross. The predicted kill temperature using the fitted curve was

$$198.19 * (10)^{(-0.0572))} = 173.7\ ^{o}\text{ Celsius} \qquad \text{(Equation 10)}$$

The temperature range was chosen to incrementally march the spores toward their kill threshold within the limits of the error that accompanies the temperature measurement.

The result of this experiment provided additional data to augment the earlier findings. Below is a repeat of the previous plots with this additional inactivation point. The results agree with the previous relationship curve. See figures 27 and 28 below.

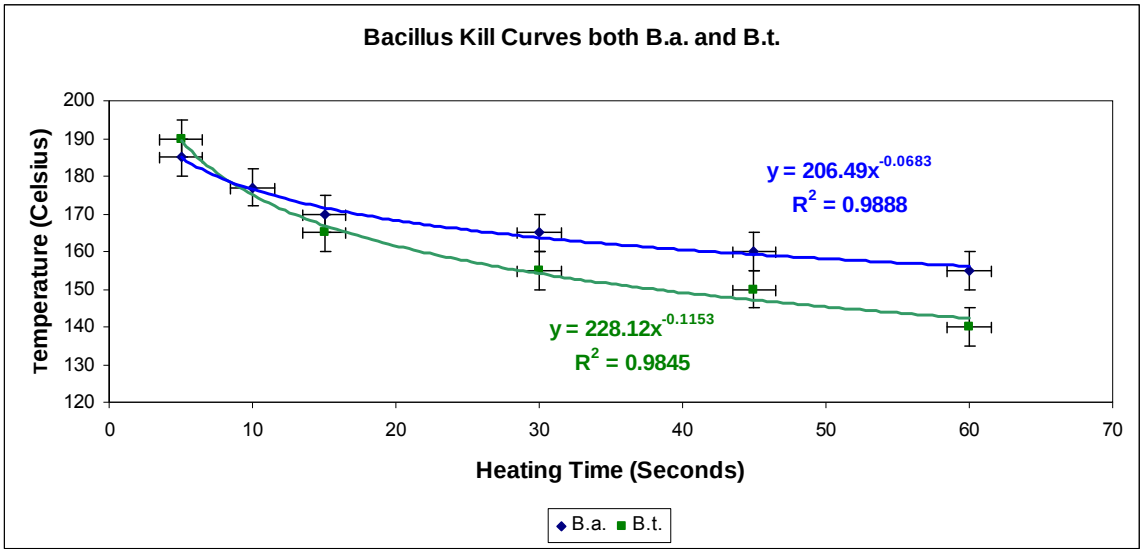

Figure 27. *Bacillus* Kill Curves of both *B.a.* and *B.t.* with additional data point for *B.a.* at the 10 second point. The additional point improves the fitness of data from $R^2 = 0.9777$ to $R^2 = 0.9888$.

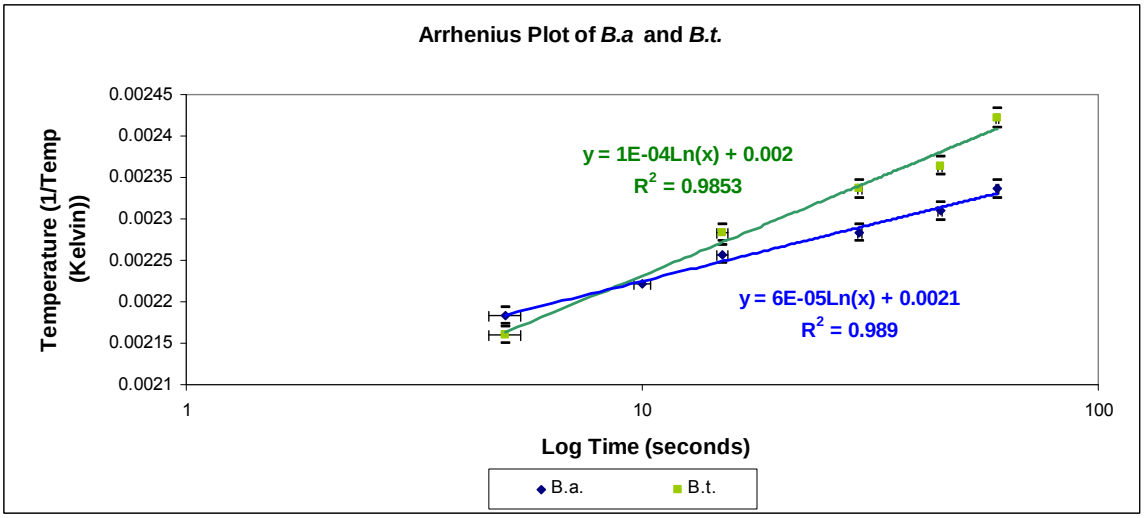

Figure 28. *Bacillus* Kill Curves of both *B.a.* and *B.t.* plotted in the Arrhenius style with additional data point for *B.a.* at the 10 second point. The additional point improves the fitness of data from $R^2 = 0.9679$ to $R^2 = 0.989$.

Additionally, a blank sample was also tracked over this incubation period that was not exposed to any heat and was considered normal growth.

Growth was measured by counting cells taken from random sample of holes across the cover slip. Data on growth was taken in 30 minute intervals after the 3 hour incubation time. Figure 29 below shows the results for surviving spores growing out of the holes. The individual vegetative cells are visible. Each cell was counted as a member of specific genetic line. Each genetic line was counted and assumed to originate from one parent spore. With this method of counting, only spores that germinated were counted. At least twenty holes per data set were sampled. The number of total genetic lines was recorded as well as the number of vegetative cells per each genetic line.

All data was collected by visual microscope inspection as determined by the observer. This method requires some subjective determinations of both genetic lines and number of vegetative cells present in each line. Looking at the example growth in Figure 29, the data recovered from this sampling would be recorded as seen in Table 2.

Table 2. Growth Data Sampling Illustration

Genetic Line	1	2	3	4	5	6	7	8
Number of cells	8	8	4	2	2	2	4	2
Total Number of Germinating Spores	8							
Total Number of Vegetative Cells	32							
Error (s)	± 2.62							

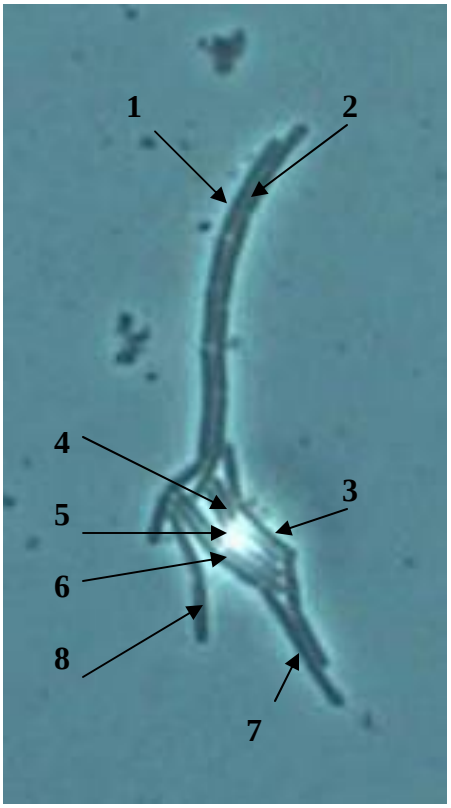

Figure 29. Measurable Spore Growth in Hole. The generations appear as links in the chain. The growth originates in the sink point of the etched hole and grows out. In this picture you can also see the shadowed edge where the hole begins with a gradual slope well outside the center sink. The numbers correspond to the sampling example shown in Table 2.

A manual count of each vegetative cells originating from a hole on the cover slip was taken at 30 minute time interval. The number of vegetative cells per linear cell line for each time was then averaged. The standard deviation among the growth was also determined for each temperature set at each time interval. Any data point outside one standard deviation length of the average was dismissed as an anomalous outlier. The numerical results are displayed in Table 3.

Table 3. Growth Data for *B.a.*

Temperature in º Celsius	Incubation time 180 minutes: Average Generation Growth	± σ	Incubation time 210 minutes: Average Generation Growth	± σ	Incubation time 240 minutes: Average Generation Growth	± σ
No Heat	2.66	1.78	5	3.3	5.306	1.06
161	0	0	1.5714	0.79	2.625	2.28
165	0	0	0	0	0	0
169	0	0	0	0	0	0
173	0	0	0	0	0	0
177	0	0	0	0	0	0

Temperature in º Celsius	Incubation time 270 minutes: Average Generation Growth	± σ	Incubation time 300 minutes: Average Generation Growth	± σ	Incubation time 360 minutes: Average Generation Growth	± σ
No Heat	7.522	3.56	7.607	8.94	9.5	4.18
161	3.6	1.71	4.07	1.55	6.625	2.53
165	3	2.07	3.5	1.63	6.38	2.48
169	0	0	2.04	0.75	6.28	2.6
173	0	0	1.17	0.73	2.076	0.64
177	0	0	0	0	0	0

This data is consolidated in Figure 30. The growth recovery curves are all shown in descending temperature order below the normal growth expectation. The higher the temperatures the longer the spores took to start to grow at all. Eventually, those spores exposed to lower than 173 º Celsius appeared to recover and eventually returned to a normal growth. The spores exposed to 173 º Celsius where the threshold point was predicted did eventually begin to grow, but the rate of growth was obviously slowed considerably.

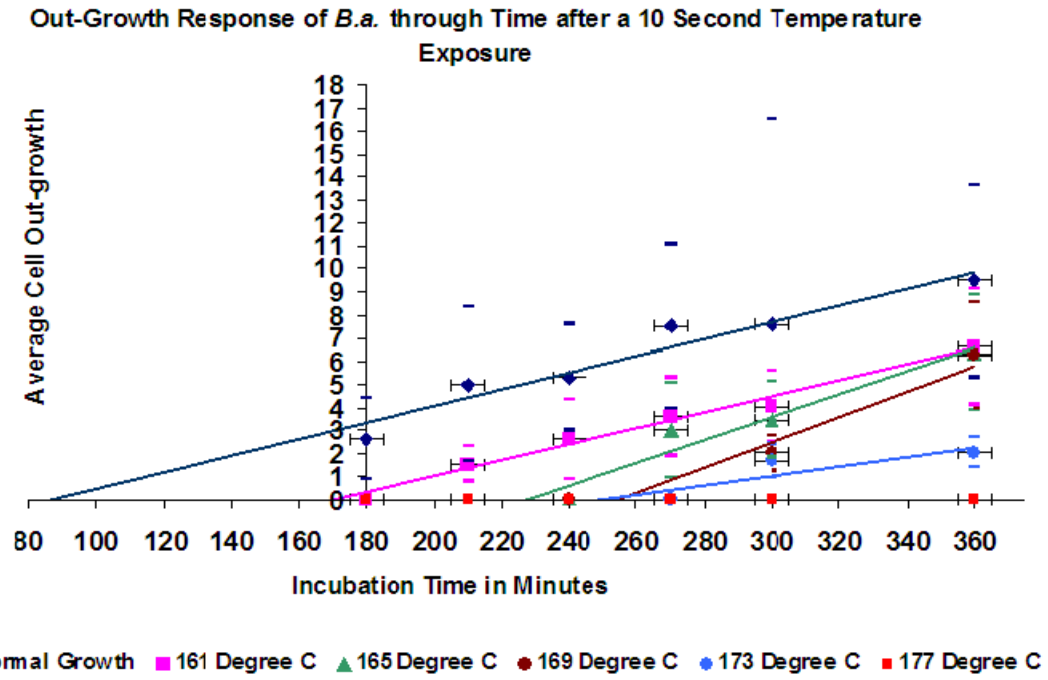

Figure 30. Growth Response Plot. Here is the consolidated data from the growth measurement shown in Table 2. The top curve is the unexposed sample blank that records normal growth rate for the *B.a.* spores prepared for these experiments. The descending curves show the injury recovery rate for each temperature. At exposure to 177 ° Celsius the spores were unable to recover. The errors in cell outgrowth are displayed as small bars with colors corresponding to their respective point. The error regions overlap significantly and indicate that the points in many cases are not statistically distinguishable.

The error for the growth data shown in Figure 30 and Table 2 was separately calculated for each point. Since the data was collected as a random sample and each sample had a variable number of spores that germinated, the total number of germinated spores counted had to be considered in the error. The error was calculated using the estimated standard deviation (s) of a finite set of data as defined by Equation 11.

$$s = \sqrt{\frac{\sum x_i^2 - \left(\left(\sum x_i \right)^2 / N \right)}{N - 1}} \qquad \text{(Equation 11)}$$

where

- x_i is the raw data values
- x_i^2 is the squared raw data values
- N is the number of data points in the included data set

Data points that were outside the 2.5s region for each data set were then eliminated based on the results. The remaining data can be viewed with a 99% confidence interval.

In addition to the error impact, there is other information not available because of experimental time constraints. The growth was monitored starting at the 180 minute point. As depicted in Figure 30 above, the growth of the unheated spores had already started. The actual growth point can only be predicted using the trend lines. Also since the growth measurement stopped at six hours, there is a possibility that the spores treated at 177 º Celsius would eventually recover viability and germinate at a later time.

Next, the trend lines for each temperature data set were used to estimate the point where germination started. On Figure 30, this is the point where the trend lines cross the x-axis. These estimated points were plotted in the Arrhenius plot style using the log of reciprocal absolute temperature exposure. The Arrhenius model predicts that the time it takes for the spore to repair the damage caused by exposure to heat should appear as a linear relationship. This relationship is indeed seen in Figure 31.

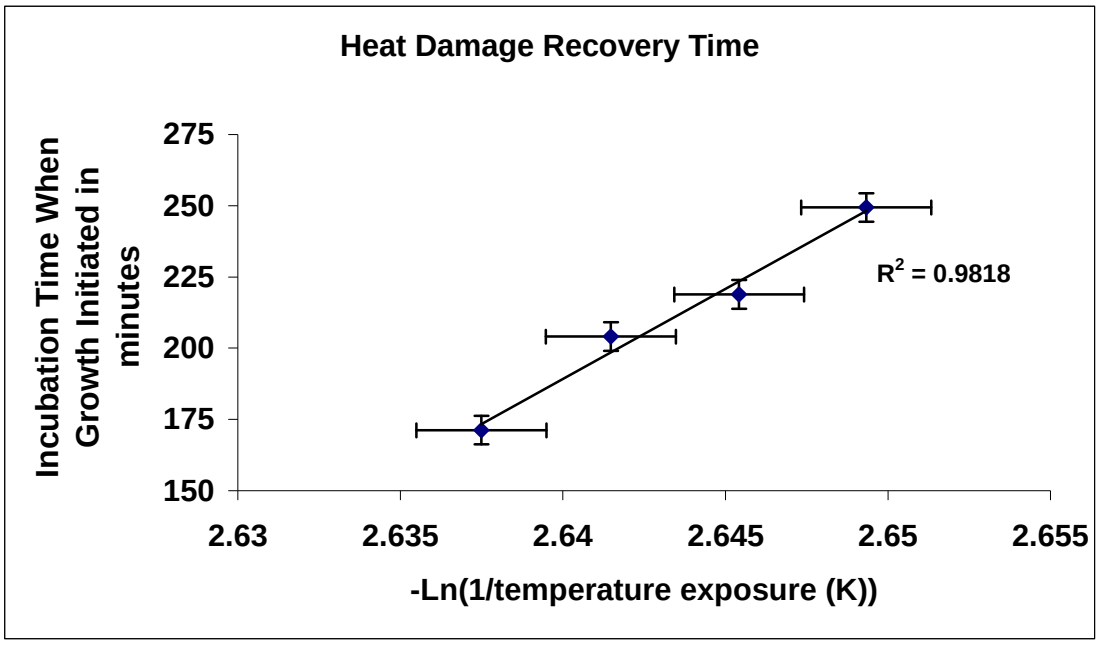

Figure 31. Heat Damage Recovery Plot. Here is the estimated initial growth point data calculated from Figure 30. The heat damage recovery shows a linear relationship as predicted by the Arrhenius model.

This resulting compilation of data was reliably obtainable through the use of the micro etched platform. The use of the current hole separation dimensions would need to be adjusted further apart if this counting method would be employed further than the six hour incubation time after which overgrowth of vegetative cells would again obscure view of healthy growth.

V. Conclusions and Recommendations

Conclusions

The main effort during the course of this research was the development and proofing of the micro-etched cover slip platform. The ultimate goal of developing a platform that would limit the spore distribution to one per hole was never reached. At the final state of development a decision between using the 3x3 micron sized mask and the larger 4x4 micron mask. The trade off of this decision affected the type of data that could be gathered. The larger 4x4 micron holes were a magnitude more successful in capturing and holding spores. However, the amount of spores in the holes was consistently greater than one. This fact hindered statistical efforts to draw conclusions. The 3x3 micron holes held less spores in each hole, but, over all was much less successful in holding any spores at all. This made statistical analysis of probability of kill unreliable.

The use of the platforms to separate the spore growth and allow an un-obscured view of each vegetative cell was fundamental in paving the way to get reliable data on growth rate. The space provided for the cells to germinate supported their natural tendency to grow in straight lines. Without the over lap and bunching, counting of generations proved much easier than experience with the traditional diluted spread plate method. To this end, the etched platforms were successful.

The other goal of this research was to validate the accepted surrogate relationship among *Bacillus* spores. The data supports the substitution of *B.t.* for *B.a.* in these type of

heating experiment, provided the two species of *Bacillus* are prepared in exactly the same manner.

Recommendations for Future Work

Ideally, additional experimentation would discovery an optimal hole dimension and removal technique that would assure a one hole - one spore ratio. Once this criterion is met the potential for statistical analysis dramatically increases. Future work could then determine the probability of kill, injury, and spore recovery.

More research is also needed to explore the effect of spore preparation factors such as water content. Moisture content has long been proven to be a factor in mortality to all organisms. Additional research could focus on comparison of moisture content to temperature response at multiple time intervals.

Finally, the area of research concerning thermal inactivation of *Bacillus anthracis* that is most deficient in information is the spore's response to high temperatures at time intervals under a second. The difficulty in these short time interval experiments seemed to lie with an inability to find a heat source that would reliably generate almost instantaneous heat. Great inroad in solving this issue was made by K. Goetz in her work to develop a laser heating method that meets this short time heating problem (Goetz; 2005). A combination of this laser heating technique and this micro-etched platform would be ideal for gathering data in this untapped region of heat inactivation.

Appendix: Tested Methods

The intent of this appendix was record methods that were tested during the course of this experiment, but ultimately failed to meet designated criteria. This information is included as valuable mistakes that can be avoided during future work involving *B.a.* and *B.t.* spores on glass cover slips. The methods discussed includes spore preparation, spore application, and spore removal.

Preparation and Application of Spores to the Cover Slips

The goal of the project was to get *B. a.* spores into the manufactured holes. Finding the best method to accomplish this took several attempts. The final application technique had to address both the problem of putting spores down on the cover slip and getting a spore into a hole as well, as the second problem of removing the remaining spores that were not in the holes, off of the surface.

The first attempt explored the traditional method of spreading a mixture of diluted spores and sterilized water. Sterile water was pipetted onto the culture plate covering the plate surface. Next, the spores were mixed with the water using a glass spread rod until a thick film was visible above the agar base. This film was then pipetted into a plastic centrifuge tube and the more sterile water was added to bring the volume to 1/3 full. The tube was vortexed to completely mix the spores before spinning for 10 minutes at 10,000g. After this step, the excess supernatant that rose to the top of the tube was poured off. More sterile water was added to again bring the volume to 1/3 full. These

last three steps were repeated two more times to essentially wash the spores ensuring that any extracellular debris was removed.

This spore solution was then pipetted onto a cover slip. After careful observation of this traditional method a glaring problem was obvious. This technique would not accomplish the first goal of putting spores in holes. The problem was simply a ratio issue. The amount of area on the cover slip that was a hole compared to the amount of surface area that was flat is less than 5%. Since the amount of spores in the solution were deliberately reduced through dilution, the probability of a spore finding a hole on the surface was plainly improbable.

In order to increase the odds created by the surface ratio, more spores needed to be present on the surface. To this end, the second method explored eliminated the calculated dilution steps. Spores were lifted from the agar growth plate using a sterile wire loop and placed directly on cover slip. Next, 0.5 to 1 milliliter of sterile water was dropped on the cover slip over the spores. The spores and water were mixed directly on the cover slip surface to form a liquid paste consistence. This spore paste was spread over the holes using a sterile wood tooth pick. The water on the spore laden cover slips was then allowed to evaporate. Since *B.a.* spores are not motile on their own, the evaporation of the water forced the spores to settle directly on the cover slip surface. This also eliminated the possibility of the surface tension of the water molecules potentially capping the holes thereby preventing spores from reaching the holes. Figure 32 below, shows this step. Next in Figures 32 and 33 are pictures that show that the spore paste employed by this method was thick enough to indeed cover the surface.

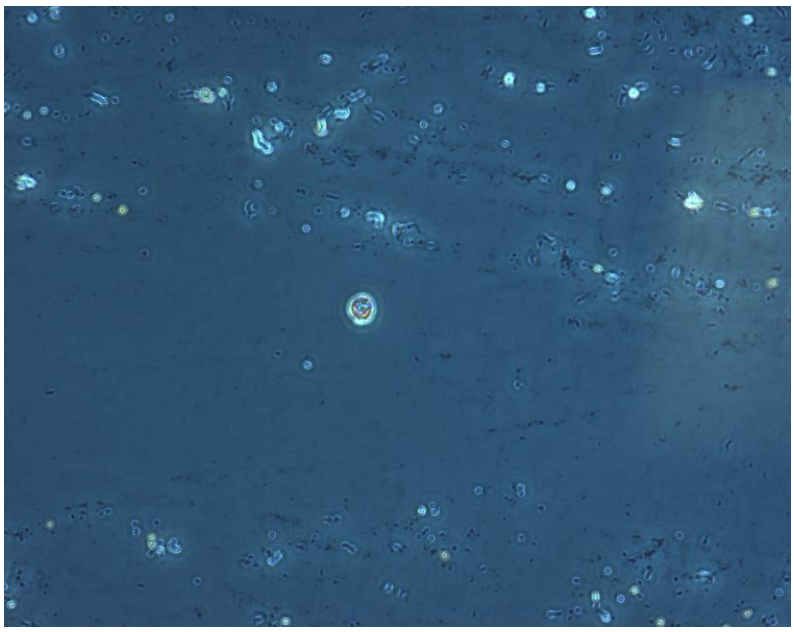

Figure 32. Slide Surface After Rinsing with Water. This is a picture taken at 200x magnification of the spore laden slide after a water rinse. Excess spores clearly remain on the surface.

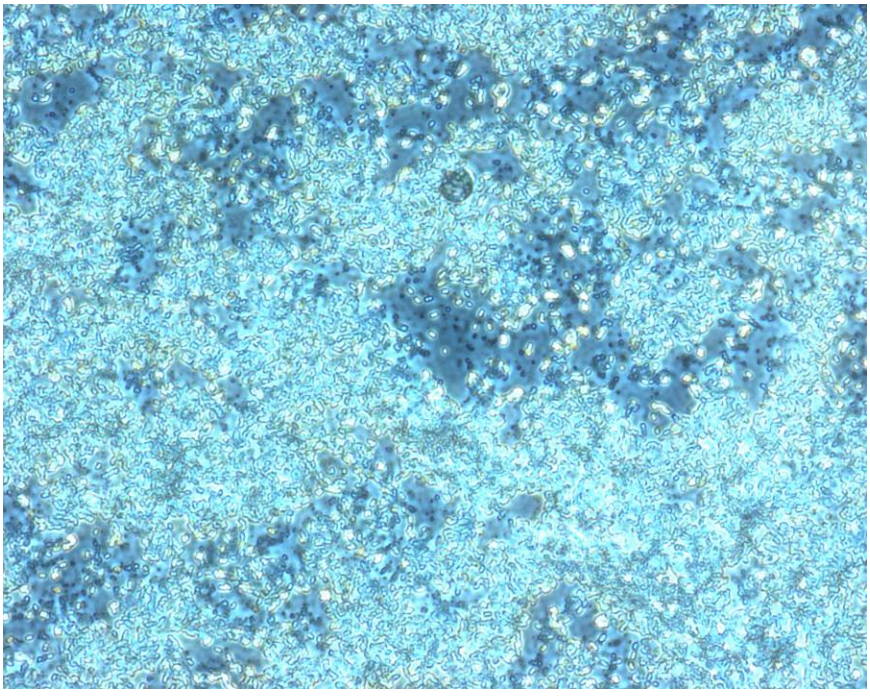

Figure 33. Wet Spore Paste above Hole. This picture taken at 200x magnification shows the suspended spores moving over the holed surface. Since spores are not motile, their movement depended of the Brownian motion of the water as it evaporated.

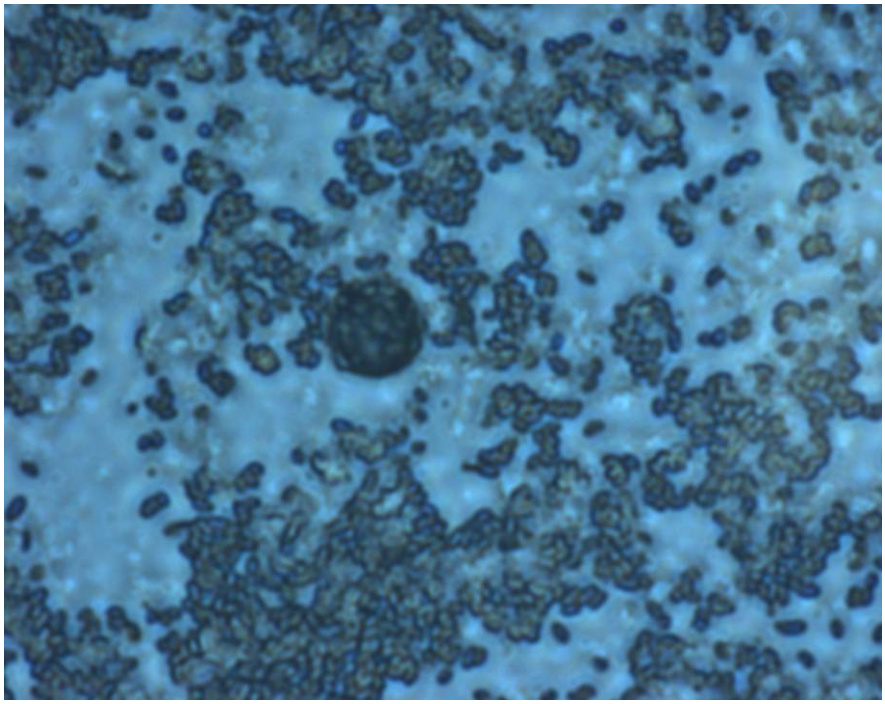

Figure 34. Evaporated Spore Smear. This picture taken at 1000x magnification shows the large concentration of spores on the surface. This also demonstrates the relative size ratio of a spore to the hole.

This method of direct application of the spores without washing manifested itself as a problem after the SEM verification. The first set of SEM pictures clearly showed a large amount of cellular debris that accompanied the spores on to the cover slips, see Figure 35, below. This debris posed several problems. First, large debris filled the holes preventing spores from getting inside. Second, the debris prevented accurate counts of numbers of spores in holes by obscuring the view of spores and allowing a place for the spores to hide. Finally, the excess debris provided additional surface areas for the spores to attached and be removed. The removal of the debris, while maintaining a high concentration of spores, resulted in the spore slurry preparation discussed in the main document.

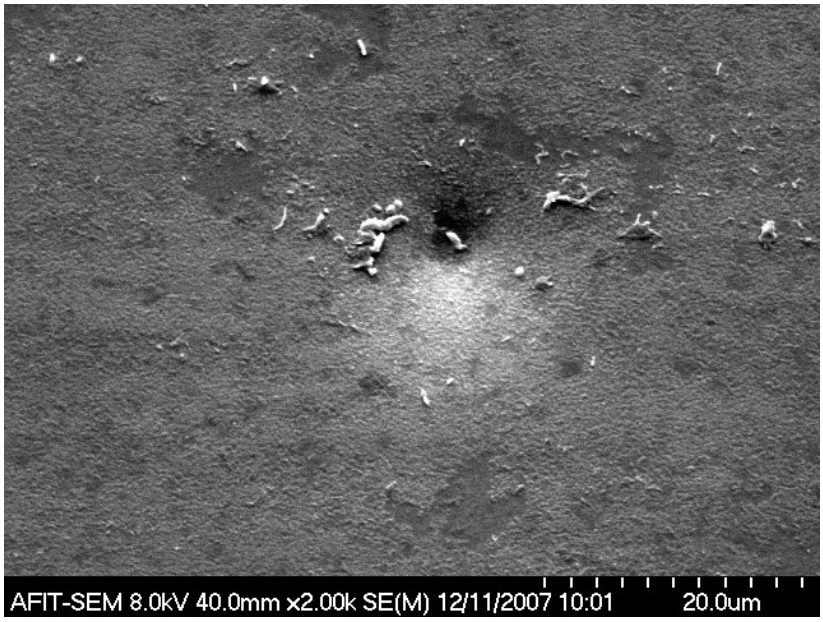

Figure 35. SEM Picture of Cellular Debris. The SEM picture reveals that presence of excess cellular debris. This debris clogged the holes, hid spores, and interfered with accurate counting of spores in holes.

Removal of Spores from the Cover Slips

Cleaning all of the extra spores from the surface was the next step in sample preparation. The trick to this process was to remove the surface spores while avoiding disturbing those spores already in the holes. This also took several attempts to solve.

The initial idea was to remove these spores by essentially mechanically scraping the surface clean. A sterile razor edge was used to applied to the surface with even pressure was swept across the spore film covered area of the cover slip. Although this method did remove most of the surface spores, it created a subsequent problem. Complete spore removal took several razor runs over the surface. Even though a new sterile razor edge was used for each run, any imperfections or unevenness of the surface released shards of glass when the razor edge passed. These shards provided additional cutting edges, which when picked up with the excess spore on successive razor runs,

scratched and marred the surface. Because the *B.a*. spores are only about a micron in diameter, the grooves made by these glass shards served as additional trenches where extra spores would get lodged, thereby making the surface harder to clean. Figure 36, below, shows the result of this scaring.

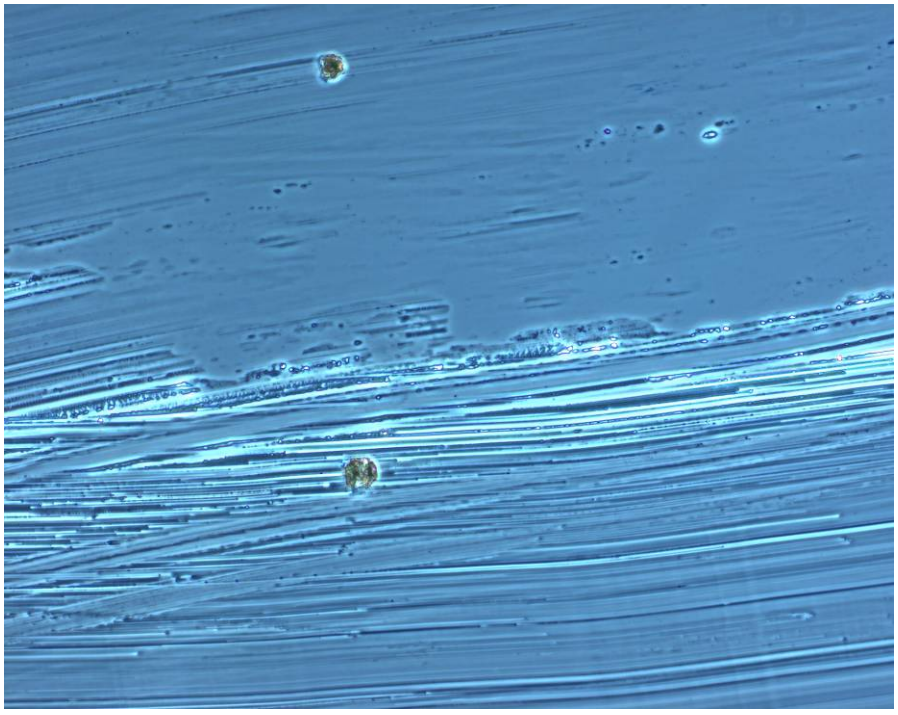

Figure 36. Razor Damage to Surface. The damage to the surface caused by glass debris caught under the razor edge is clearly visible.

A gentle approach worked much better. Instead of scraping the spores off the surface with a hard edge, the spores were rinsed off with sterile water. The cover slips were rinsed with the water until the spore paste film was no longer visible. The spores that remained on the surface after the rinsing were then wiped away with clean laboratory chem-wipes. Since the surface was moist, the spores were lifted by the wipes without damaging the surface like the razor did. Figure 37 shows a cover slip prepared with this rinse and wipe technique. After numerous spore germination trials this method was

chosen as the final technique for applying spores to the cover slips. It was actually easier to perform and yielded consistent results in both numbers of occupied holes and surface cleanliness.

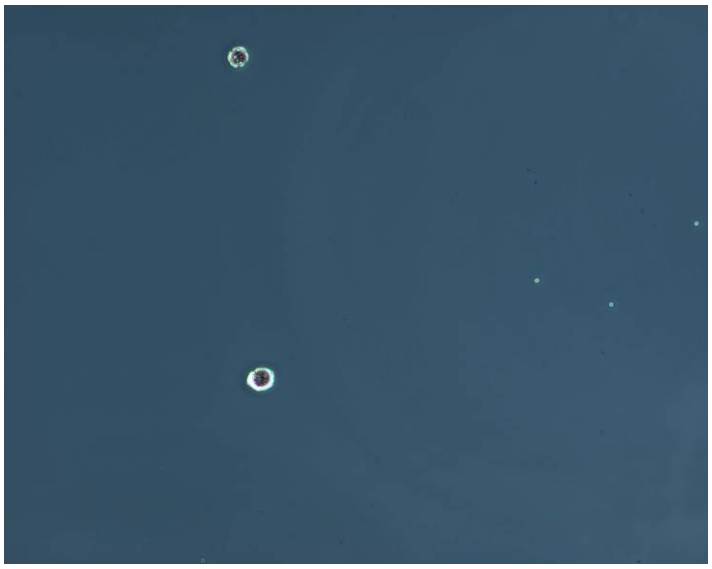

Figure 37. Rinsed and Wiped Surface. The surface was rinsed with sterile water until there was no visible film on the surface. Then successive surface wiping with Chem.-Wipes® was repeated until the surface was clear of spores. The mottled, dark areas of the holes suggest the presence of spores.

Figure 38. Verified Surface Success. Each successive technique was tested by germinating the spores to see if the technique achieved the criteria of spores in holes while surface is clear. This is a successful demonstration of the discussed rinse and wipe technique.

This removal technique worked with the direct spore application method, but once the spores were cleaned, this method failed. The application of the spores directly from the agar plate also applied some agar and the aforementioned cellular debris. Once this extra material was removed, the spores were more predispositioned to be rinsed off the surface and absorbed by the Chem.-Wipe®. This meant that the removal method for the washed spore slurry had to be dry. The combination of needing a flat edged that would not scratch like the razor, was strong enough to ply the spores off the surface, but would also not absorb water led to the used of the plastic weigh boat.

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14. ABSTRACT

This research developed and applied microlithography techniques using a microscope slide platform in order to measure thermal inactivation of B.t. and B.a. spores, two closely related bacillus species. B.t. is widely used as a substitution for B.a. in response studies and as a B.a. surrogate in some field studies. This work addressed the previously identified problem of measuring both spore growth and spore thermal kill threshold using the traditional method of spreading a diluted spore solution on a plate. The micro-etched platforms forced spore separation thereby preventing neighbor growth from obscuring germination and initial vegetative growth measurements. The technique permits observation of small samples of spores over time and yielded more accurate response measurements. This study includes comparison of thermal responses between B.a. and B.t. spore species that were prepared and stored in exactly the same environment and conditions. Findings support the continued use of B.t. as a substitute for B.a. in this type of work and especially in studies for thermal inactivation for short times periods of a minute or less.

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